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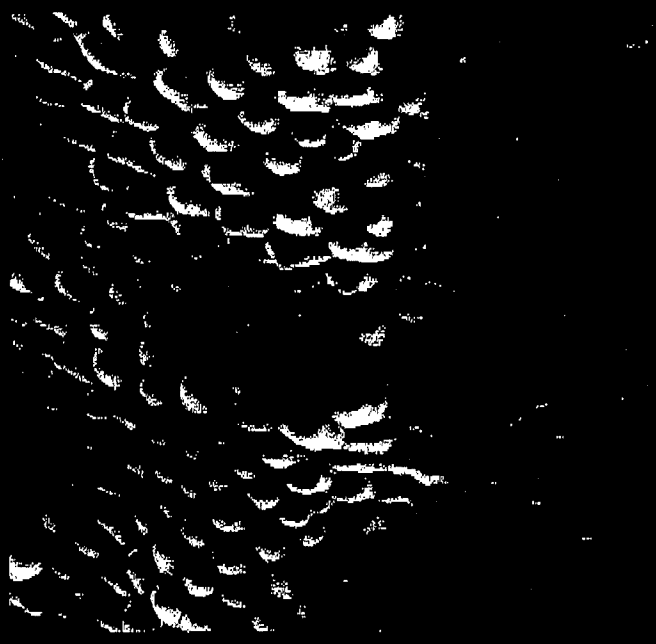

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RAFT1: A Mammalian Protein That Binds to FKBP12 in a Rapamycin-Dependent Fashion and Is Homologous to Yeast TORs

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Summary

The immunosuppressants rapamycin and FK506 bind to the same intracellular protein, the immunophilin FKBP12. The FKBP12–FK506 complex interacts with and inhibits the Ca^{2+}-activated protein phosphatase calcineurin. The target of the FKBP12–rapamycin complex has not yet been identified. We report that a protein complex containing 245 kDa and 35 kDa components, designated rapamycin and FKBP12 targets 1 and 2 (RAFT1 and RAFT2), interacts with FKBP12 in a rapamycin-dependent manner. Sequences (336 amino acids total) of tryptic peptides derived from the 245 kDa RAFT1 reveal striking homologies to the yeast TOR gene products, which were originally identified by mutations that confer rapamycin resistance in yeast. A *RAFT1* cDNA was obtained and found to encode a 289 kDa protein (2549 amino acids) that is 43% and 39% identical to TOR2 and TOR1, respectively. We propose that RAFT1 is the direct target of FKBP12–rapamycin and a mammalian homolog of the TOR proteins.

Introduction

The natural products cyclosporin A, FK506, and rapamycin are potent immunosuppressants with realized or potential clinical applications in the prevention of graft rejection after organ transplantation and the treatment of autoimmune disorders (Borel, 1986; Kino et al., 1987; Martel et al., 1977). These drugs act by inhibiting intermediate steps in the signaling pathways that effect the T-cell response to antigen (for reviews, see Fruman et al., 1994; Kunz and Hall, 1993; Liu, 1993; Schreiber, 1991). This makes them useful probes for identifying the components of those pathways and determining their physiological roles.

The immunosuppressants interact with the immunophilins, which are small, soluble, receptor proteins that mediate their actions. Cyclosporin A (a cyclical undecapeptide) binds to cyclophilin A, whereas FK506 and rapamycin (two related macrolide antibiotics) bind to a distinct receptor protein, FKBP12 (Handschumacher et al., 1984; Harding et al., 1989; Siekierka et al., 1989). Though cyclophilin and FKBP12 differ markedly in amino acid sequence, both immunophilins have peptidyl–prolyl cis–trans isomerization (rotamase) activity, which is inhibited by their respective ligands (reviewed by Heitman et al., 1992). However, this inhibition does not appear to explain the effects of the immunosuppressants (Bierer et al., 1990a, 1990b; Tropschug et al., 1989). Instead, the action of cyclosporin A and FK506 derives from the binding of the drug–receptor complexes to the calcium-activated protein phosphatase calcineurin (Liu et al., 1991). This association inhibits the catalytic activity of the phosphatase, which is required for the Ca^{2+}-dependent initial step in the activation of the T lymphocyte via the T cell receptor (Flanagan et al., 1991; Kronke et al., 1984).

On the other hand, rapamycin appears to block a later, Ca^{2+}-independent stage in the T cell response. This drug selectively inhibits the IL-2 stimulated G1 to S cell cycle transition that initiates T cell proliferation (Dumont et al., 1990b). Although this inhibition has been correlated with the decreased activity of the 70 kDa S6 kinase ($pp70^{S6K}$), a known downstream effector of the IL-2 receptor, the FKBP12–rapamycin complex does not appear to interact directly with this kinase (Chung et al., 1992; Kuo et al., 1992). Similarly, in T cells and other cell types, rapamycin blocks progression of the cell cycle by preventing the activation of the cyclin-dependent kinases $p33^{cdk2}$ and $p34^{cdc2}$, but an association of the drug–immunophilin complex with the kinases or their respective cyclins has not been demonstrated (Albers et al., 1993; Jayaraman and Marks, 1993; Morice et al., 1993).

In the budding yeast Saccharomyces cerevisiae, rapamycin also causes an arrest in the G1 phase of the cell cycle through its binding to a highly conserved FKBP12 homolog (Heitman et al., 1991b). The toxicity of the drug for yeast cells has allowed, through genetic selection, the identification of two homologous genes, which, when mutated, render the cells rapamycin-resistant (Heitman et al., 1991a). This led to the proposal that the products of these genes, which show some amino acid homology to the catalytic domain of the p110 subunit of PI-3 kinase, are the targets of rapamycin, hence the designation of the genes as TOR1 and TOR2 (Kunz et al., 1993). Direct support for this proposal, however, has not been presented, and how the TOR gene products confer sensitivity to rapamycin remains to be elucidated. Alternatively, it has been suggested that in the signaling pathway blocked by rapamycin, the TOR proteins, like the S6 kinase and the cyclin-dependent kinases, lie downstream of the direct target of the FKBP12–rapamycin complex (Albers et al., 1993; Helliwell et al., 1994). This model assumes that the *TOR* mutations lead to the constitutive activation of the TOR1 and TOR2 proteins.

Besides binding to calcineurin in a FK506-dependent manner, FKBP12 can also interact with calcium-channel proteins, the ryanodine receptor, which mediates calcium induced calcium release (Jayaraman et al., 1992; Timerman et al., 1993), and the inositol 1,4,5,-triphosphate (IP_3) receptor (A. Cameron, A. Kaplin, D. M. S., J. Steiner, and S. H. S., unpublished data). These associations do not require FK506 or rapamycin; indeed, these drugs dissociate the FKBP12–channel complex.

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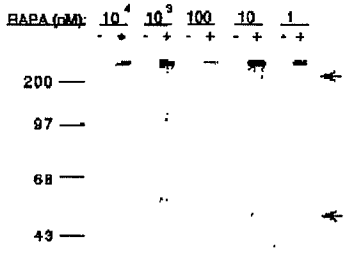

Figure 1. Rapamycin-Dependent Cross-Linking of FKBP12 to Two PC12 Cell Cytosolic Proteins of Approximate Molecular Weight 245 kDa and 35 kDa

^{32}P-labeled FKBP12 (10^6 cpm) was incubated with cytosolic fractions from PC12 cells with or without the indicated concentration of rapamycin for 1 hr at 4°C. The cross-linker DSS was then added and the incubation continued for 40 min before processing for SDS–PAGE (4%–12% gradient) and autoradiography. The arrows indicate the two bands that appear only in the presence of rapamycin. This experiment was repeated three times with identical results.

In this paper we have sought to identify, through cross-linking and affinity purification procedures, cellular protein(s) that interact with FKBP12 only in the presence of rapamycin. We report the identification of two such proteins, of approximately 245 and 35 kDa, which we designate rapamycin and FKBP12 targets 1 and 2, or RAFT1 and RAFT2, respectively. The amino acid sequence of RAFT1 displays extensive sequence similarities to the predicted sequences of yeast TOR1 and TOR2.

Results

Rapamycin Promotes the Binding of FKBP12 to Two Cytosolic Proteins of M, 245 and 35 kDa

A ^{32}P-radiolabeled *FKBP12* probe was used to detect proteins that associate with the immunophilin in the presence of ligand and are cross-linked to it by the bivalent reagent disuccinimidyl suberate (DSS). The probe was prepared by phosphorylating with [γ-^{32}P]ATP a recombinant rat *FKBP12* to which two consensus sites for cyclic AMP-dependent protein kinase (PKA) were added at the N-terminus (Blanar and Rutter, 1992; Li et al., 1992). Since this modification did not alter the capacity of the protein to associate with calcineurin in the presence of FK506 (data not shown), the probe can be used to identify a target of the FKBP12–rapamycin complex.

PC12 pheochromocytoma cell cytosolic extracts were incubated with ^{32}P-FKBP12 in the presence or absence of rapamycin and then treated with the cross-linker DSS before gel electrophoretic analysis, followed by autoradiography. The drug caused the formation of two protein complexes with radioactive FKBP12, corresponding to bands of M, 260 and 50 kDa (Figure 1). Taking into account the 15 kDa M_r of the modified *FKBP12* probe, the cross-linked proteins were estimated to be 245 kDa and 35 kDa, respectively. The cross-linked complexes were observed over a wide rapamycin concentration range, but were more prominent at the low concentrations of 1 and 10 nM, possibly because of an inhibitory effect on the association of the higher amounts of ethanol (the solvent of the drug) present at the higher drug concentrations (Figure 1). Rapamycin also induced the formation of similar complexes when the probe was incubated with cytosolic extracts from several rat tissues, including liver, kidney, heart, small intestine, thymus, testes, spleen, and brain (data not shown), but no significant differences in abundance of the cross-linked proteins between the tissues were observed. For convenience, further experiments were carried out with whole-brain extracts.

The formation of the rapamycin-dependent complexes was specific for FKBP12, since in similar experiments with the related immunophilin ^{32}P-FKBP25, no ligand-induced complexes were observed (data not shown).

Specificity of the Rapamycin-Induced Association: The Interaction of ^{32}P-FKBP12–Rapamycin with the 245 and 35 kDa Proteins Is Competed by FK506 and by Unlabeled FKBP12

To investigate further the specificity of the interaction of ^{32}P-FKBP12–rapamycin with the cytosolic proteins, we performed a partial purification to remove endogenous FKBP12, which is present in brain at high concentrations (Steiner et al., 1992). This was accomplished by chromatography on a heparin column, to which the cytosolic proteins that interact with FKBP12–rapamycin bound and could be eluted at 300–450 mM KCl (Figure 2). Free FKBP12, on the other hand, was recovered in the flowthrough of this column, as demonstrated by binding to [^{3}H]FK506 (data not shown).

FK506 antagonizes actions of rapamycin, and both drugs compete for the same binding site on FKBP12 (Bierer et al., 1990a; Dumont et al., 1990a). Accordingly,

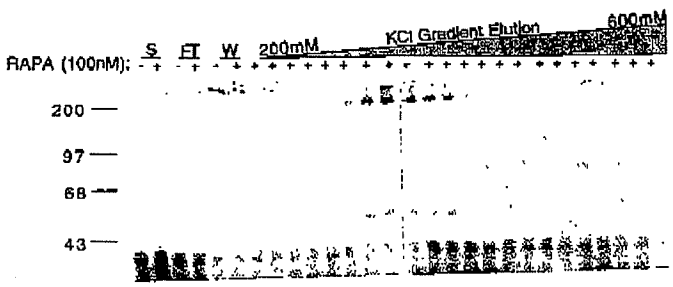

Figure 2. Partial Purification of the FKBP12–Rapamycin Target Proteins from Brain Cytosol by Heparin Column Chromatography

A cytosolic fraction prepared from a rat brain homogenate was applied to a heparin column. The material that remained bound to the column after washing with 5 column vol of wash buffer (see Experimental Procedures) containing 200 mM KCl was eluted with a linear gradient from 200 mM to 600 mM KCl in homogenization buffer. Aliquots of the crude cytosol (S), the column flow through (FT), and the wash (W) were tested in the cross-linking assay with (plus) or without (minus) rapamycin (100 nM). Every other fraction eluted from the heparin column was tested in the cross-linking assay in the presence of 100 nM rapamycin. No rapamycin-epecific cross-linked products are visible in the crude cytosol, owing to the high concentrations of endogenous FKBP12 present in the initial sample.

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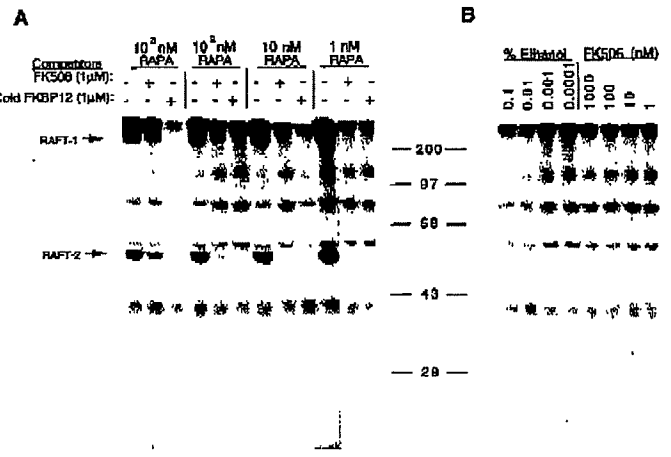

Figure 3. FK506 and Unlabeled FKBP12 Prevent the Rapamycin-Dependent Association of ^{32}P-FKBP12 to the Target Proteins

(A) The heparin column eluate containing the RAFTs was tested in the cross-linking assay at the indicated concentrations of rapamycin with or without the addition of 1 μM FK506 or 1 μM FKBP12.

(B) Neither FK506 alone nor the ethanol vehicle induces cross-linking of FKBP12 to RAFT. The heparin eluate containing RAFT was tested in the cross-linking assay with the indicated concentrations of FK506 or ethanol. This experiment was repeated twice with identical results.

we examined the influence of FK506 on the rapamycin-induced interaction of ^{32}P-FKBP12 with its putative cytosolic targets. At concentrations ranging from 1 nM to 1 μM, rapamycin induced the appearance of intense bands representing cross-linked proteins and, at all rapamycin concentrations tested, this effect was antagonized by 1 μM FK506 (Figure 3A). As expected for ligands of similar affinity for FKBP12, when equal concentrations (1 μM) of rapamycin and FK506 were present, the intensities of the cross-linked bands were reduced by approximately 50%, and the reduction progressively increased with increasing ratios of FK506/rapamycin. The heparin column eluate apparently contains limiting amounts of the putative targets of the FKBP12–rapamycin complex, since excess unlabeled FKBP12 (1 μM) completely suppressed the appearance of the cross-linked bands containing labeled FKBP12 (Figure 3A).

Control experiments (Figure 3B) confirmed the specificity of the rapamycin effect, since the formation of the complex was not induced by several concentrations of FK506 or by ethanol, the solvent of the drugs. These experiments demonstrate that the cross-linked proteins are specific targets of the FKBP12–rapamycin complex and not of the FKBP12–FK506 complex, nor of FKBP12 alone. Therefore, we designate the cross-linked proteins RAFT1 (245 kDa) and RAFT2 (35 kDa).

We attempted to separate RAFT1 and RAFT2 under nondenaturing conditions by several chromatography and gel filtration procedures, including DEAE and CM cellulose, reactive dye green 5, and Superose 6 (data not shown). All of these efforts failed, suggesting that RAFT1 and RAFT2 are part of a complex, although it is possible that RAFT2 is a proteolytic fragment of RAFT1 that contains the FKBP12–rapamycin-binding site and remains tightly bound to the rest of the polypeptide.

Purification of RAFT1

We purified RAFT1 from the heparin column eluate on the basis of its affinity for FKBP12–rapamycin. We constructed a glutathione S-transferase (GST)–FKBP12 fusion protein by cloning, in-frame downstream of GST, a cDNA encoding FKBP12 with two N-terminal PKA consensus sites (Smith and Johnson, 1988; Blanar and Rutter, 1992; Li et al., 1992). The encoded protein was expressed in bacteria, purified, and immobilized on glutathione–agarose beads. SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the beads recovered after incubating them with the heparin eluate in the presence or absence of rapamycin shows that the drug induces the binding to the beads of a protein of 245 kDa (Figure 4). With this simple purification scheme, we were able to purify about

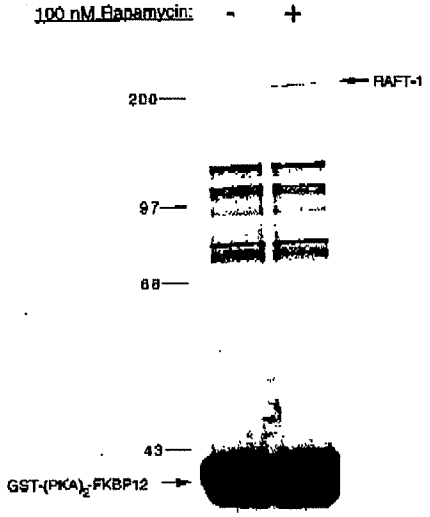

Figure 4. Purification of RAFT1 with a FKBP12–Rapamycin Affinity Column

RAFT-enriched fractions eluting from the heparin column between 300 and 450 mM KCl were incubated in the presence (plus) or absence (minus) of 100 nM rapamycin with GST–(PKA)2–FKBP12 fusion protein (20 μg) immobilized on glutathione–agarose beads. The material that remained associated with the beads after extensive washes was analyzed by SDS–PAGE (6%) and silver staining. RAFT1 is present only in the sample treated with rapamycin. The large band at 36 kDa is the GST–FKBP12 fusion protein.

5 μg of RAFT1. A low transfer efficiency to nitrocellulose membrane resulted in only 2.5 μg being available for protein sequencing, which corresponds to 10 pmol of a protein of this size.

Whether RAFT2 was also bound to the beads could not be determined in this experiment, because its presence would be masked by the large band of similar M_r corresponding to the GST–$(PKA)_2$–FKBP12 fusion protein. When smaller fusion proteins, such as an epitope-tagged FKBP12, were employed for the affinity matrix, the binding of the 35 kDa RAFT2 could also be observed (data not shown).

Protein Sequencing of RAFT1: Homology to TOR1 and TOR2

Affinity-purified RAFT1 was separated by SDS–PAGE from other proteins that adsorbed to the glutathione–agarose beads, transferred to nitrocellulose membrane, and digested with trypsin. Fractionation of the tryptic digest by narrow-bore reverse phase chromatography yielded a complex pattern of over a hundred peaks, whose purity was assessed by mass spectroscopy. In most cases, the peaks exhibited multiple mass-to-charge peak values, and it was necessary to rechromatograph these peak fractions on a microbore column of different selectivity (see Experimental Procedures). Sequences of 23 peptides separated in this fashion were determined by a combination of automatic Edman degradation, matrix-assisted laser desorption mass spectroscopy, and ultraviolet spectroscopy.

Several protein sequence data bases (PIR, SwissProt, translated GenBank) were searched for sequences that match any of the 23 peptide sequences obtained from microsequencing of RAFT1. While sequence similarities with hundreds of different proteins were obtained for many of the 23 peptides, none perfectly matched with any of the entries in the data bases, nor did any protein match more than one or two peptides, other than the yeast proteins TOR1 and TOR2 (Kunz et al., 1993). Strikingly, 16 of the 23 peptides of RAFT1 could be aligned with the yeast TOR sequences, with varying degrees of similarity (Figure 5).

Molecular Cloning of RAFT1

To generate a probe for isolating a *RAFT1* cDNA, two degenerate oligonucleotides were used in a mixed oligonucleotide polymerase chain reaction (PCR) (Gould et al., 1989) with rat brain cDNA as template. The sense primer was made to a peptide sequence (TYDPNQP) obtained

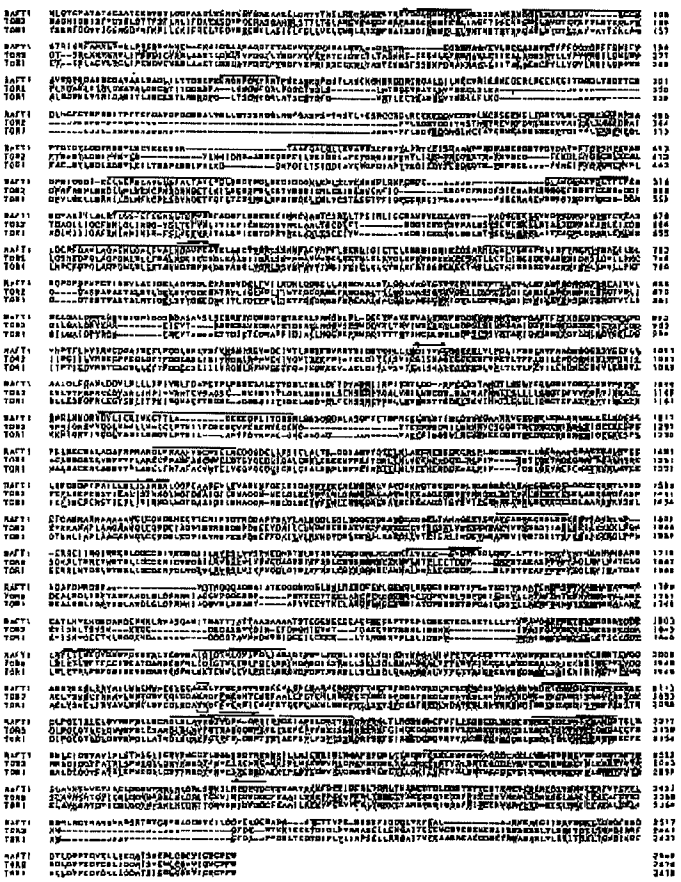

Figure 5. Alignment of RAFT1 Amino Acid Sequence with the Predicted Amino Acid Sequences of TOR2 and TOR1

The alignment was maximized by introducing insertions marked by dashes. Sequences in RAFT1 identical to TOR2, TOR1, or both are indicated with gray shading. The sequences of tryptic peptides obtained by microsequencing are indicated with a line above the RAFT1 sequence. Sequences used to design primers for PCR are indicated with an arrow above the residues (direction indicates sense or antisense). The putative PKC site conserved between RAFT1, TOR1, and TOR2 is boxed.

from microsequencing of RAFT1, while the antisense primer corresponds to a sequence (HIDFGD) conserved between TOR1, TOR2, and p110 PI-3 kinase. From the alignments of the RAFT1 peptides to the TORs, this sequence was expected to be 220 amino acids downstream of that encoded by the sense primer. The predicted 660 bp PCR product was obtained and cloned, and its authenticity was verified by DNA sequencing, which showed that it encoded two other sequenced tryptic peptides. The PCR product was, therefore, used as a probe (3′ probe) to screen a rat striatum cDNA library, which yielded a 5.5 kb partial cDNA clone. An antisense oligonucleotide to the extreme 5′ end of this cDNA was then used in a PCR reaction with a degenerate sense oligonucleotide to another peptide sequence (NDQVFE) obtained from microsequencing. The predicted 1.1 kb PCR product was obtained, cloned, and used as probe (5′ probe) to screen a rat brainstem cDNA library in parallel with the original 3′ probe. Phage plaques that hybridized with both probes were isolated, and one was found to carry a 8.5 kb insert.

This cDNA contains an open reading frame of 7.6 kb with an initiation methionine codon that conforms to the Kozak consensus sequence (Kozak, 1986) and is preceded by an in-frame termination codon. The protein encoded by this open reading frame contains all 23 peptide sequences obtained by microsequencing of RAFT1 (Figure 5). Interestingly, none of the peptides sequenced correspond to the C-terminal 250 amino acids of RAFT1, which may indicate that this portion of the protein was proteolytically removed during the purification.

The *RAFT1* cDNA predicts a protein of 2549 amino acids with a molecular mass of 289 kDa and a P_I of 6.8. Over its entire sequence, RAFT1 is 43% identical to TOR2 and 39% identical to TOR1 (Figure 5). The C-terminal 600 amino acids of RAFT1, which by analogy to the TORs (Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994) are predicted to contain lipid kinase activities, are 65% identical to the yeast proteins. The RAFT1 protein has over 20 consensus sites for phosphorylation by protein kinase C (PKC), including one at Ser-2035, which is in the analogous position to the serine (Ser-1972 in TOR1 and Ser-1975 in TOR2) found mutated to arginine in rapamycin-resistant yeast (boxed residues in Figure 5).

The predicted RAFT1 protein is 80 amino acids longer than the TOR proteins and contains several regions with no apparent homology to the yeast proteins, the largest being a 93 amino acid insertion corresponding to residues 270–363 of RAFT1. It is possible that these regions are generated by alternative splicing of exons that may be tissue specific to the brain. They are unlikely to be the translation product of unspliced introns, because they were found in several cDNA clones isolated from different libraries, and the DNA sequence does not reveal consensus splice junction sites (data not shown).

Discussion

We have isolated and identified a protein, which we designate RAFT1, that interacts with the FKBP12–rapamycin complex. Several lines of evidence suggest that the interaction between RAFT1 and FKBP12–rapamycin is responsible for the observed physiological effects of rapamycin on signal transduction pathways. Thus, in the FKBP12–rapamycin pathway RAFT1 is analogous to calcineurin in the FKBP12–FK506 pathway. Rapamycin-induced binding of FKBP12 to RAFT1 occurs at drug concentrations as low as 1 and 10 nM, resembling pharmacological potency in vivo (Bierer et al., 1990a; Dumont et al., 1990a). FK506 and rapamycin bind with similar affinities to the same binding site on FKBP12 and antagonize the actions of one another in vivo (Bierer et al., 1990a; Dumont et al., 1990b). Consistent with these facts, FK506 does not induce interactions between FKBP12 and RAFT1 but, instead, prevents the rapamycin-mediated effect. We see substantial inhibition of the interaction of FKBP12–rapamycin with RAFT1 at FK506/rapamycin concentration ratios of 10:1, which contrasts with the large excesses of FK506 (≥1000:1) necessary to inhibit the in vivo effects of rapamycin (Albers et al., 1993; Jayaraman et al., 1993; Morice et al., 1993). This discrepancy probably reflects the large amounts of intracellular FKBP12 that must be saturated by FK506 to block the access of rapamycin. In our in vitro binding assay, no endogenous FKBP12 is present, so that competition takes place at smaller excesses of FK506.

The amino acid sequence predicted from the *RAFT1* cDNA, of which 330 amino acids were confirmed by direct sequencing of tryptic peptides of purified RAFT1, reveals substantial sequence similarities between this protein and the predicted amino acid sequences of the 280 kDa yeast TOR1 and TOR2 gene products. Indeed, given the similar sizes of the proteins, RAFT1 is probably a mammalian homolog of the TOR proteins. Although our protein sequence alignments between RAFT1 and the TOR sequences indicates a slightly higher (43%–39%) identity for TOR2 than TOR1, we cannot conclusively predict from this alone whether RAFT1 is functionally equivalent to TOR2 or TOR1.

The TOR genes were initially identified as genes mutated in rapamycin-resistant yeast that also exhibit nonallelic noncomplementation with the yeast FKBP12 homolog (Kunz et al., 1993). This led to the proposal that, in yeast cells, FKBP12–rapamycin associates with the TORs and inhibits their putative lipid kinase activity (Kunz et al., 1993). Recently, the mutation in TOR1 and TOR2 that confers rapamycin resistance was identified as a serine to arginine change in a potential protein kinase C phosphorylation site in the proposed lipid kinase domain of the molecule (Helliwell et al., 1994; Cafferkey et al., 1993). This led to an alternative suggestion that the direct target of FKBP12–rapamycin is an upstream effector of the TORs that normally activates their putative lipid kinase activities and that the TOR mutations constitutively activate the proteins, bypassing the requirement for an upstream effector (Albers et al., 1993; Helliwell et al., 1994). Our demonstration of a mammalian TOR homolog as a direct target of FKBP12–rapamycin argues against this suggestion. Instead, the mutated serine, which is conserved in RAFT1, may be a structural element necessary for the immunophilin–drug complex to recognize its target. Thus, perhaps

after phosphorylation, the serine residue forms part of the recognition site for FKBP12–rapamycin, or is necessary for TORs/RAFT1 to assume the proper conformations for recognition by FKBP12–rapamycin without necessarily being part of the actual binding site.

The function(s) of RAFT1 and the TORs remains to be established. The amino acid homology of the TORs with the catalytic domain of the p110 subunit of PI-3 kinase suggests that it too is a lipid kinase (Carpenter et al., 1990; Kunz et al., 1993). The exact function of the two-subunit PI-3 kinase itself is still unknown, although it has been shown that this protein associates with several *src*-like and receptor-type tyrosine kinases, such as the PDGF, CSF-1, and IL-2 receptors, and v-*src* (reviewed by Cantley et al., 1991). The kinetics of PI-3 kinase activation are coincident with the activation of the growth factor receptors, suggesting that the 3-phosphorylated phosphoinositide products are second messengers involved in controlling cellular growth and proliferation (Whitman et al., 1988; Auger et al., 1989; Balla et al., 1994). Since rapamycin has pleiotropic effects on a wide variety of cell types, the target of its complex with FKBP12 is likely to be an early participant in several signal transduction pathways. We therefore propose that, like PI-3 kinase, RAFT1 may be an early effector in signaling pathways that are activated by cell surface receptors.

Experimental Procedures

Materials

Frozen rat brains stripped of the meninges were obtained from Harlan Bioproducts (Indianapolis, IN). Other materials were purchased from the following sources: [γ-^{32}P]ATP (NEG-002) from New England Nuclear (Cambridge, MA); glutathione–agarose, heart muscle kinase (PKA, P2645), and heparin–agarose from Sigma Chemical (St. Louis, MO); thrombin and antithrombin from Boehringer-Mannheim (Indianapolis, IN); and DSS from Pierce (Rockford, IL). Rapamycin was a gift of the Wyeth–Ayerst company (Philadelphia, PA) and FK506 a gift of the Fujisawa company (Tsukuba City, Japan).

Preparation of GST–(PKA)$_2$–FKBP12 and GST–(PKA)$_2$–FKBP25 Fusion Proteins

Standard techniques of molecular biology cloning were used as described (Sambrook et al., 1989), unless otherwise specified. All cDNAs obtained by PCR were sequenced using the Sequenase kit (Amersham, Arlington Heights, IL). cDNAs for the rat FKBP12 and FKBP25 were obtained with the PCR using 5′ and 3′ primers to the corresponding human FKBP12 (Standaert et al., 1990) or FKBP25 (Jin et al., 1992) sequences. The cDNAs were cloned into pBluescript (Stratagene, La Jolla, CA).

A 5′ primer (PKA-12-1 or PKA-25-1) encoding a BamHI site, two consensus PKA phosphorylation sites (Blanar and Rutter, 1992; Li et al., 1992), and the first six amino acids of FKBP12 or FKBP25 was used with a 3′ primer (PKA-12-2 or PKA-25-2) encoding an EcoRI site and the last six codons of *FKBP12* or *FKBP25* in a PCR with Vent Polymerase (New England Biolabs, Beverly, MA) using the rat *FKBP* cDNAs cloned in pBluescript as templates. The amplified DNA fragments were gel purified, digested with BamHI and EcoRI, and cloned into the pGEX-2T vector (Pharmacia, Upsala, Sweden) that had been linearized with the same restriction enzymes. The resulting construct was used to transformed BL21 (DE3) Escherichia coli (Novagen, Madison, WI), in which expression can be induced with IPTG.

The primer sequences were as follows: PKA-12-1, 5′-CCGGATCCCGTGGAGCTTCAGTTGAACTACGGCGTGCTTCTGTAGCCATGGGAAGTGCAGGTGGA-3′; PKA-12-2, 5′-GGCCGGAATTCTCATTCCAGTTTTAGAA-3′; PKA-25-1, 5′-CCGGATCCCGTGGAGCTTCAGTTGAACTACGGCGTGC TTCTGTAGCCATGGGCGGCGGCCGTTCC-3′; and PKA-25-2, 5′-GGCCGGAATTCTCAATCAATATCCACTA-3′.

The fusion proteins were purified with glutathione–agarose as previously described (Smith and Johnson, 1988), from bacterial cultures induced with 1 mM IPTG.

Labeling of the Immunophilin Fusion Proteins Containing N-Terminal Phosphorylation Sites for PKA

The fusion proteins were labeled with a modification of published procedures (Blanar and Rutter, 1992; Li et. al., 1992). Purified GST–PKA–FKBP12 or GST–PKA–FKBP25 (10 mg) was mixed with 40 U of PKA and 100 mCi of [γ-^{32}P]ATP in a buffer containing 20 mM Hepes (pH 7.7), 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT.

After 1.5 hr at 37°C, the incubation mixture containing labeled fusion protein was dialyzed twice against 1 liter of thrombin cleavage buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2.5 mM $CaCl_2$). The labeled fusion protein was cleaved by adding an equal volume of thrombin cleavage buffer containing 2 mg/ml thrombin and incubating at room temperature for 2 hr. The thrombin was inactivated by adding an equal volume of a stop solution consisting of 1 mM DTT, 1 mM PMSF, 100 U/ml antithrombin III. The specific activity of the probes was estimated at 1×10^6 cpm/pmol of the protein.

Cell Culture

PC12 cells were maintained in culture as described (Altin et al., 1991).

Preparation of Tissue or PC12 Cell Extracts

Fresh or frozen whole rat brains were homogenized with a polytron in 5 ml per brain of homogenization buffer consisting of 20 mM Hepes (pH 6.8), 150 mM KCl, 1 mM EGTA, 0.1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 4 mM DTT, 1 mM PMSF, and protease inhibitors (4 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml pepstatin, 2 μg/ml chymostatin). Heavy membranes and nuclei were removed by centrifugation at 35,000 × g for 30 min. The supernatant was made 1 mM $MgCl_2$ and 0.1% CHAPS before further purification.

PC12 cells were lysed in homogenization buffer with 0.3% NP-40 instead of CHAPS. Lysis was accomplished in 2 ml of buffer per T-150 flask by repeated vortexing at 4°C. Cell debris was sedimented by centrifugation at 10,000 × g for 10 min at 4°C.

Cross-Linking Assay

The labeled, cleaved FKBP12 was diluted to 10,000 cpm/ml in 50 mM Hepes (pH 7.5), 1 mg/ml BSA. Labeled protein (10 μl; 100,000 cpm total), 10 μl of tissue or PC12 cell extract, and 10 μl of drug diluent buffer (20 mM Hepes [pH 6.8], 100 mM KCl, 1 mM EGTA, 1 mM DTT) containing either 3-fold the desired final concentration of rapamycin, FK506, or equivalent amounts of ethanol, were mixed and incubated for 1 hr at 4°C. After this incubation, 1 μl of 5.6 mg/ml DSS was added and the incubation continued for 40 min. The reaction was terminated by adding one column volume of 2× concentrated sample buffer (Laemmli, 1970) containing 50 mM Tris (pH 7.4) and processed by SDS–PAGE (10%, unless otherwise specified) and autoradiography.

Heparin Column Chromatography and Affinity Column Purification

The rat brain extract was applied to a heparin column (2 ml of packed heparin–agarose per brain) at a flow rate of 1.5 ml/min. The column was washed with 10 column volumes of buffer (20 mM Hepes [pH 6.8], 200 mM KCl, 1 mM EGTA, 50 mM NaF, 1.5 mM Na_3VO_4, 4 mM DTT, 1 mM PMSF) and the same protease inhibitors as in the homogenization buffer. The material bound to the column was eluted with a linear KCl gradient from 200 to 600 mM in homogenization buffer. Aliquots (10 μl) of the fractions collected were tested in the cross-linking assay, and positive fractions were pooled and concentrated in a Centriprep-100 (Amicon, Beverly, MA) to 1/3 starting volume. The flowthrough of the heparin column was assayed for the presence of FKBP with a [^{3}H]FK506 binding assay, as described (Steiner et al., 1992).

The concentrated heparin column eluate was incubated for 2 hr at 4°C with 1/50 volume of glutathione–agarose to remove endogenous glutathione-binding proteins. The beads were removed by centrifugation at 1000 × g for 3 min. Fresh glutathione–agarose (1/500 volume) and 20 μg of purified GST–PKA–FKBP12 fusion protein were then added to the cleared heparin column eluate with or without 100 nM rapamycin. After a 1 hr incubation at 4°C, the beads were washed 5× with 1.5 ml of ice-cold PBS containing 1% Triton X-100 and 500

mM NaCl. The beads were transferred to 3× volume SDS–PAGE sample buffer, the eluted proteins were fractionated by SDS–PAGE, and the gel was silver stained.

For protein sequence analysis, affinity-purified material derived from 50 brains was fractionated by SDS–PAGE and transferred to nitrocellulose membranes. The proteins transferred were visualized by Ponceau S staining and the 245 kDa RAFT1 band excised and processed for internal amino acid sequence analysis, essentially as described (Tempst et al., 1990).

Protein Sequence Analysis

Membrane-bound protein, about 2.5 μg, was subjected to in situ proteolytic cleavage using 1 μg of trypsin (sequencing grade; Boehringer-Mannheim) in 25 ml of 100 mM NH_4HCO_3 (supplemented with 10% acetonitrile and 3% Tween 80) at 37°C for 3 hr. The resulting peptide mixture was reduced and S-alkylated with, respectively, 0.1% β-mercaptoethanol and 0.3% 4-vinyl pyridine, and fractionated by two-dimensional reversed phase HPLC.

For the primary separations, a 2.1 mm Vydac C4 (214TP54) column was used with gradient elution at a flow rate of 100 μl/min. HPLC solvents and system configuration were as described (Tempst et al., 1990), with improved dead volume reduction through the use of glass capillary tubing (C. Elicone and P. T., unpublished data). Identification of Trp-containing peptides was done by manual ratio analysis of absorbances at 297 and 277 nm, monitored in real time using an Applied Biosystems model 1000S diode array detector (Tempst et al., 1990). Fractions were collected by hand, kept on ice for the duration of the run, and then stored at −70°C before repurification, analysis, or both. An enzyme blank was done on an equally sized strip of nitrocellulose cut from a blank area of the same blot. Repurifications (second dimension LC) were carried out on a 1.0 mm SGE ODS-2 C18 column using the same solvent system but at a flow rate of 30 μl/min (Elicone et al., 1994). Samples were always acidified (20% TFA final concentration) and then diluted 2-fold with 0.1% TFA before rechromatography.

Peak fractions over background were analyzed by a combination of automated Edman degradation and matrix-assisted laser desorption (MALDI-TOF) mass spectrometry (Geromanos et al., 1994; Elicone et al., 1994). After storage, column fractions were supplemented with neat TFA (to give a final concentration of 10%) before loading onto the sequencer disc and mass spectrometer probe tips. Peptide mass analysis (on 2% aliquots) was carried out using a model LaserTec Research MALDI-TOF instrument (Vestec), with a 337 nm output nitrogen laser and 1.2 m flight tube. The matrix was α-cyano-4-hydroxy cinnamic acid, and a 28 kV ion acceleration and 4.3 kV multiplier voltage were used. Laser power and number of acquisitions were adjusted as judged from optimal deflections of specific maxima, using a Tektronix TDS 520 digitizing oscilloscope. Mass-to-charge spectra were generated from the time-of-flight files using GRAMS data analysis software. Every sample was analyzed twice, in the presence and absence of a calibrant (25 fmol APID), as described (Geromanos et al., 1994). Chemical sequencing (on 95% of the sample) was done using a model 477A instrument from Applied Biosystems. Stepwise liberated PTH–amino acids were identified using an online 120A HPLC system (Applied Biosystems) equipped with a PTH C18 (2.1 × 220 mm; 5 μm particle size) column (Applied Biosystems). Instruments and procedures were optimized for femtomole level phenyl thiohydantoin amino acid analysis as described (Tempst and Riviere, 1989; Erdjument-Bromage et al., 1993).

Peptide average isotopic masses were summed from the identified residues (including the presumed ones) using ProComp version 1.2 software (obtained from Dr. P. C. Andrews, University of Michigan, Ann Arbor, MI). Peptide sequences were compared with entries in various sequence data bases using the National Center for Biotechnology Information BLAST program (Altschul et al., 1990). Lower stringency alignments between all peptides and selected proteins were done using the Lipman–Pearson algorithm, available in the Lasergene software package (DNASTAR).

Isolation of cDNA Clones

A degenerate sense oligonucleotide corresponding to the amino acid sequence TYDPNQP, which was obtained from microsequencing of RAFT1 and aligns to residues 2086–2093 of TOR2, and a degenerate antisense primer corresponding to amino acids 2296–2301 (HIDFGD) of TOR2, were used in a PCR reaction with rat whole-brain cDNA as template. The protocol for the PCR was as follows: an initial 5 min at 94°C, followed by 35 cycles of 94°C for 40 s, 56°C for 1 min, 72°C for 1 min, and a final incubation at 72°C for 5 min. The PCR products were fractionated on a 1.1% agarose gel and the expected 700 bp DNA fragment purified and subcloned into pBluescript.

The *RAFT-1* cDNA fragment in pBluescript was amplified by PCR and the product gel purified and labeled by nick translation with a commercial kit (Boehringer-Mannheim). This probe (designated 3′ probe) was used to screen 10^6 phage plaques of a rat striatum λZAP library (Stratagene), as described (Sambrook et al., 1989). Of 47 positive clones identified, 10 were purified by an additional two rounds of screening. None of the inserts contained a complete open reading frame. The 5′ end of the largest insert (5.5 kb) was used to design a 18 bp antisense oligonucleotide (3.1as) that was used in another PCR reaction with rat whole-brain cDNA as template and a degenerate oligonucleotide corresponding to the amino acid sequence NDQVFE (part of a peptide obtained from microsequencing) as the sense primer. The PCR products were fractionated on a 1% agarose gel and a DNA fragment of 1.1 kb isolated and cloned into the vector pCR-II using the TA cloning kit (Invitrogen, San Diego, CA). The cDNA fragment was amplified by PCR and the product gel purified and labeled by nick translation. This probe (designated 5′ probe) was used to screen 10^6 phage plaques from a rat brainstem λZAP library. Duplicate filters were screened with the 3′ probe. Eight clones hybridized with both the 5′ and 3′ probes, and five of these were purified through two additional rounds of screening. One clone contained an 8.6 kb insert that encodes all 23 peptide sequences obtained by microsequencing.

PCR primer sequences were as follows: TYDPNQP, 5′-GGGGGATCCACNTA(C/T)GA(C/T)CCNAA(C/T)CA(A/G)C-3′; HIDFGD, 5′-GCGGAATTC(G/A)TCNCC(G/A)AA(G/A)TC(T/G/A)AT(G/A)TG-3′; NDQVFE, 5′-GGGGGATCCAA(C/T)GA(C/T)CA(G/A)GTNTT(T/C)GA-3′; and 3.1as, 5′-GAGCCACCACGATTTGCT-3′.

cDNA clones were sequenced using the fluorescent terminator method of cycle sequencing on an Applied Biosystems 373a automated DNA sequencer at the DNA Analysis Facility of the Johns Hopkins University (Smith et al., 1986; McCombie et al., 1992), or with the dideoxy chain termination method using the Sequenase kit (Amersham, Arlington Heights, IL). Oligonucleotides used for sequencing were synthesized on an ABI 394 synthesizer following ABI protocols. DNA sequence data was analyzed using Sequencher software from Gene Codes (Ann Arbor, MI). Protein alignments were done with help from the e-mail service of the Computational Biochemistry Research Group at the Eidgenoessische Technische Hochschule.

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GenBank Accession Number

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(12) **United States Patent**
Sabatini et al.

(10) **Patent No.: US 6,492,106 B1**
(45) **Date of Patent: Dec. 10, 2002**

(54) **MAMMALIAN PROTEINS THAT BIND TO FKBP12 IN A RAPAMYCIN-DEPENDENT FASHION**

(75) Inventors: **David M. Sabatini**, Baltimore, MD (US); **Hediye Erdjument-Bromage**, New York, NY (US); **Mary Lui**, Kew Gardens, NY (US); **Paul Tempst**, New York, NY (US); **Solomon H. Snyder**, Baltimore, MD (US)

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(21) Appl. No.: **08/305,790**

(22) Filed: **Sep. 14, 1994**

Related U.S. Application Data

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(51) **Int. Cl.**[7] **C12Q 1/68**

(52) **U.S. Cl.** **435/6**; 536/23.1; 536/23.4; 536/23.5; 536/24.3

(58) **Field of Search** 536/23.5, 23.4, 536/24.3, 23.1; 530/350; 435/69.1, 69.7, 91.1, 91.2, 6

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(57) **ABSTRACT**

A protein complex containing 245 kDa and 35 kDa components, designated RAFT1 and RAFT2 (for Rapamycin And FKBP12 Target) interacts with FKBP12 in a rapamycin-dependent manner. This interaction has the pharmacological characteristics expected from the observed in vivo effects of rapamycin: it occurs at low nanomolar concentrations of rapamycin and is competed by excess FK506. Sequences (330 amino acids total) of tryptic peptides derived from the affinity purified 245 kDa RAFT1 reveals striking homologies to the predicted products of the yeast TOR genes, which were originally identified by mutations that confer rapamycin resistance in yeast. A RAFT1 cDNA was obtained and found to encode a 289 kDa protein (2550 amino acids) that is 43% and 39% identical to TOR2 and TOR1, respectively.

12 Claims, 10 Drawing Sheets

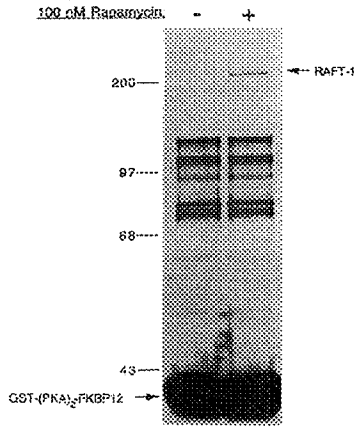

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FIG. 1

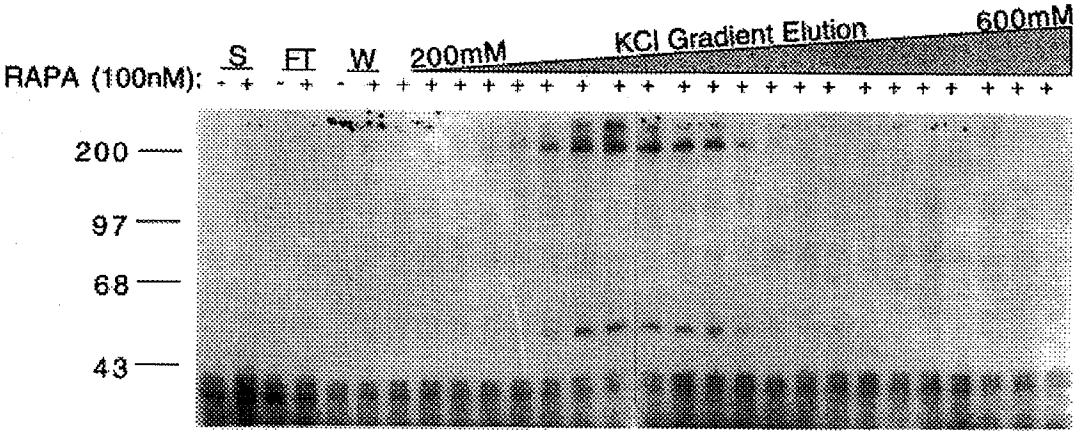

RAPA (100nM):
S
FT
W
200mM
KCl Gradient Elution
600mM
200
97
68
43

FIG. 2A

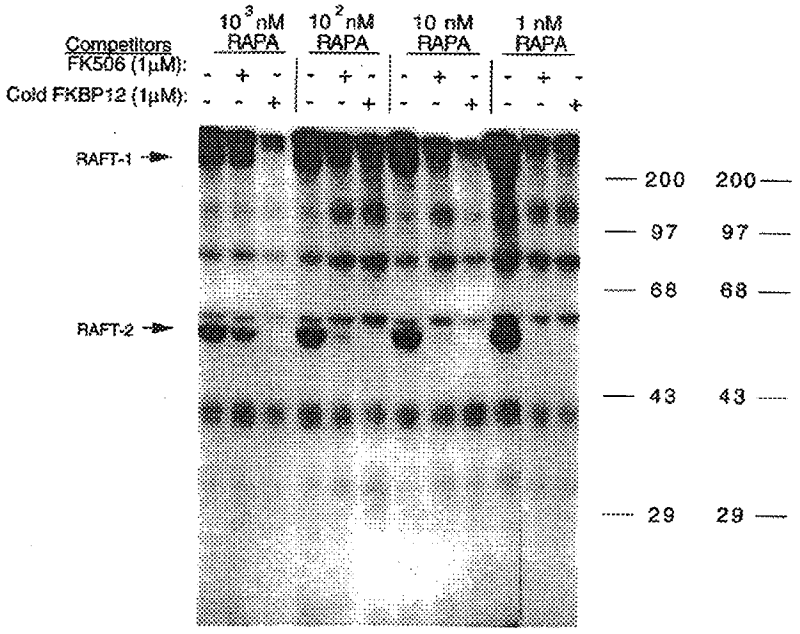

Competitors
FK506 (1μM):
Cold FKBP12 (1μM):
10³nM RAPA
10²nM RAPA
10 nM RAPA
1 nM RAPA
RAFT-1
RAFT-2
200
97
68
43
29

FIG. 2B

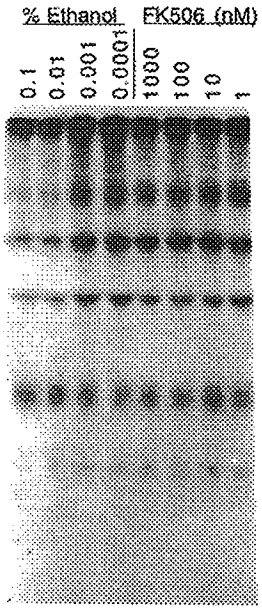
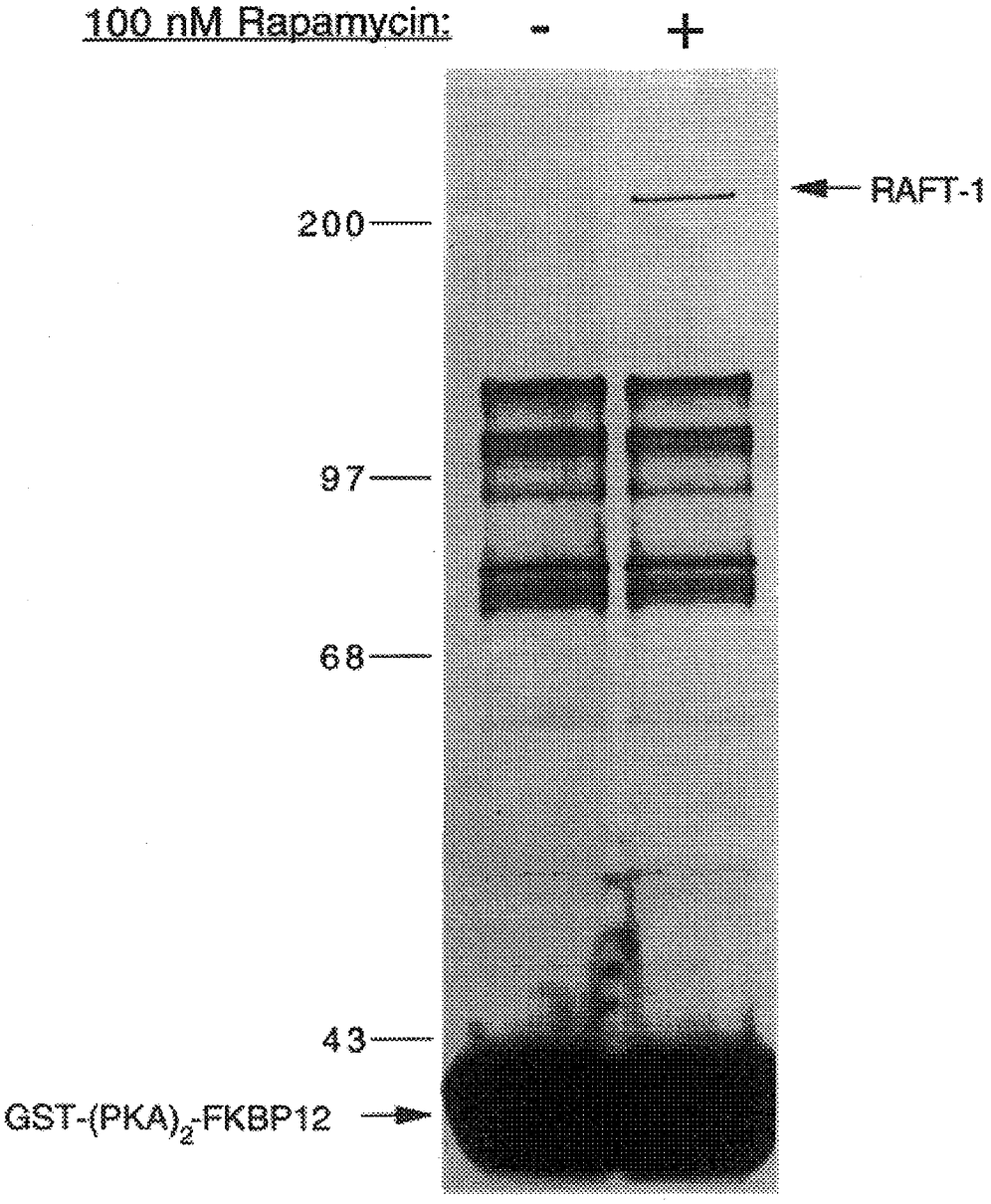

% Ethanol
FK506 (nM)
0.1
0.01
0.001
0.0001
1000
100
10
1

FIG. 3



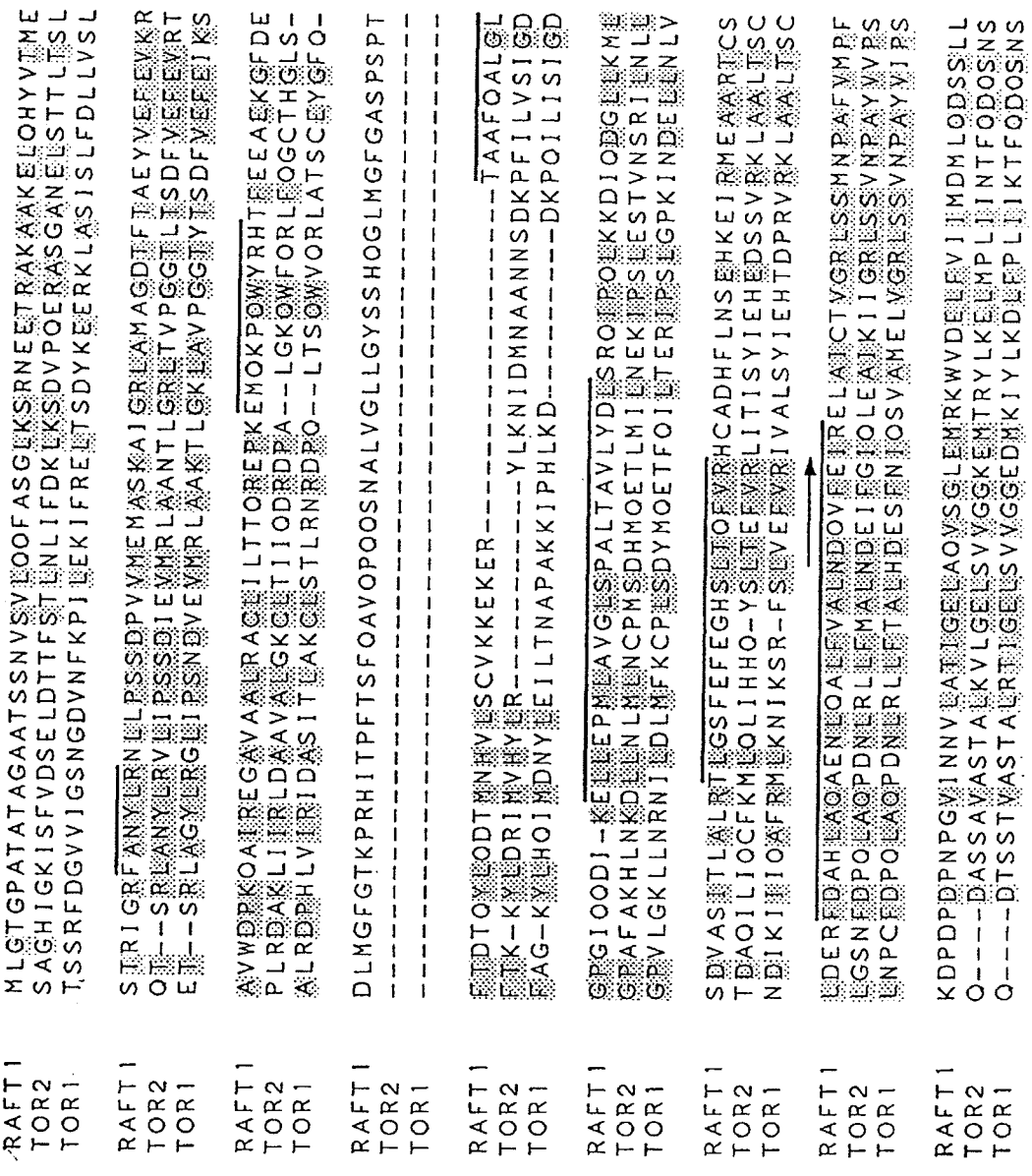


FIG. 4A

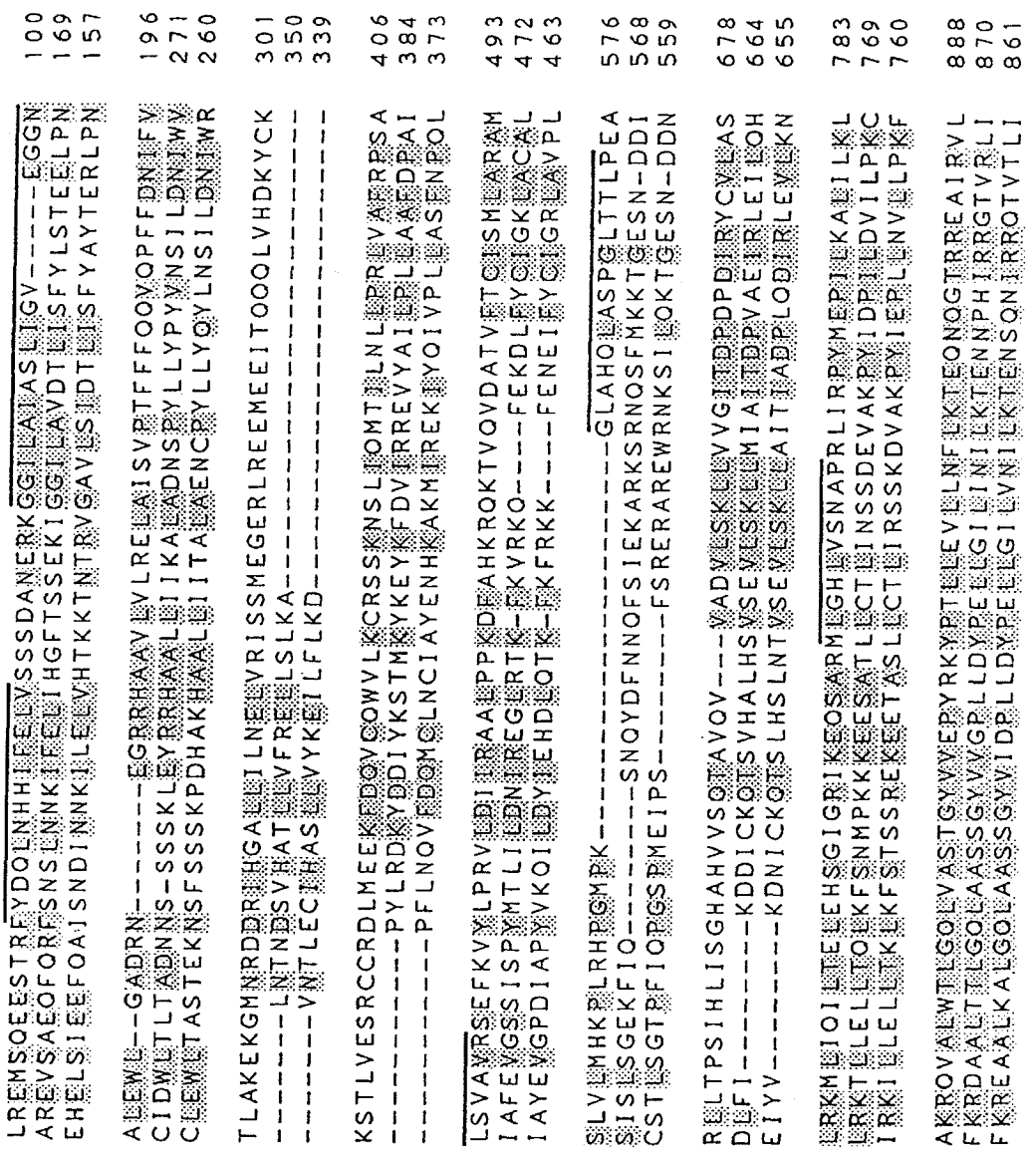


FIG. 4B

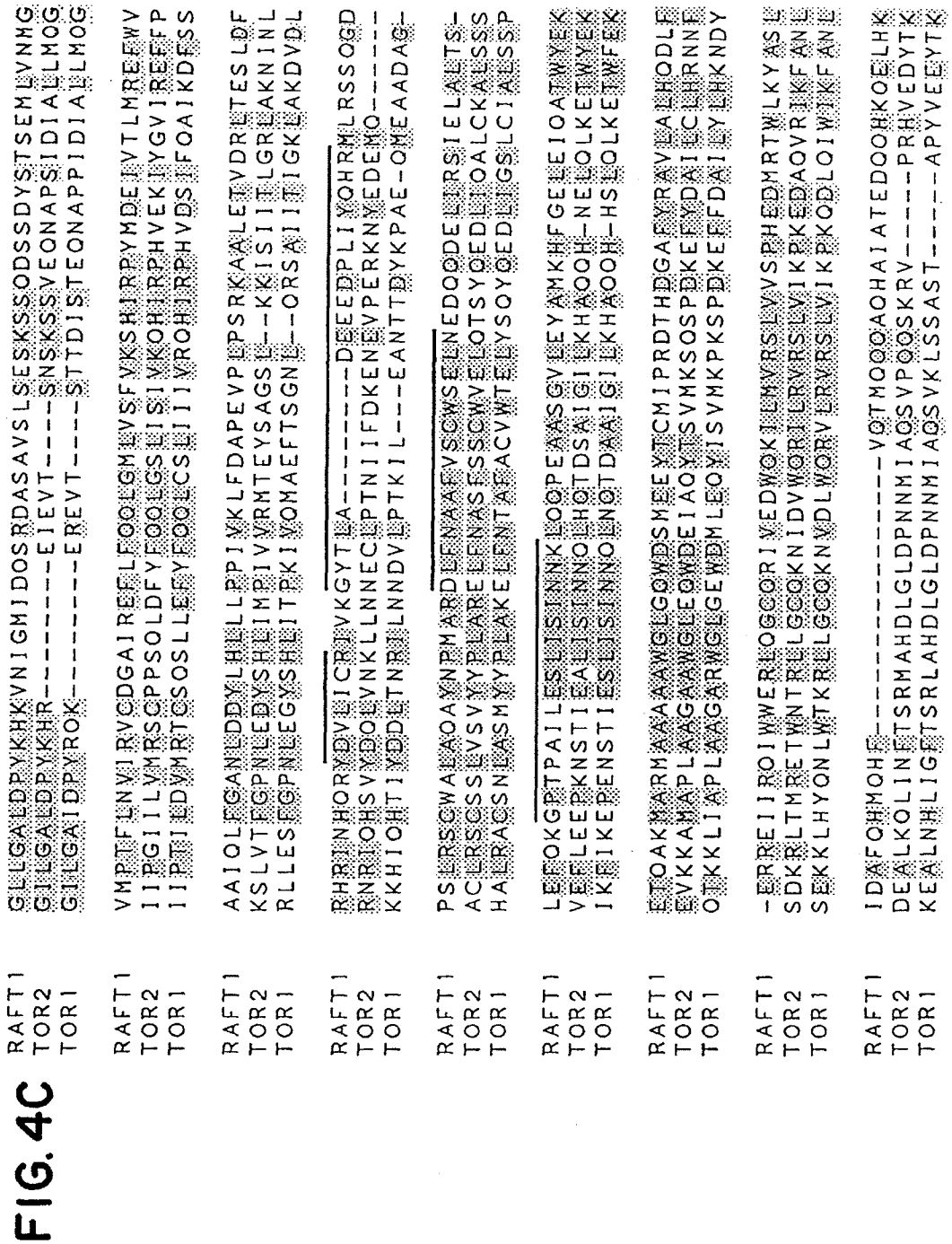


FIG. 4C

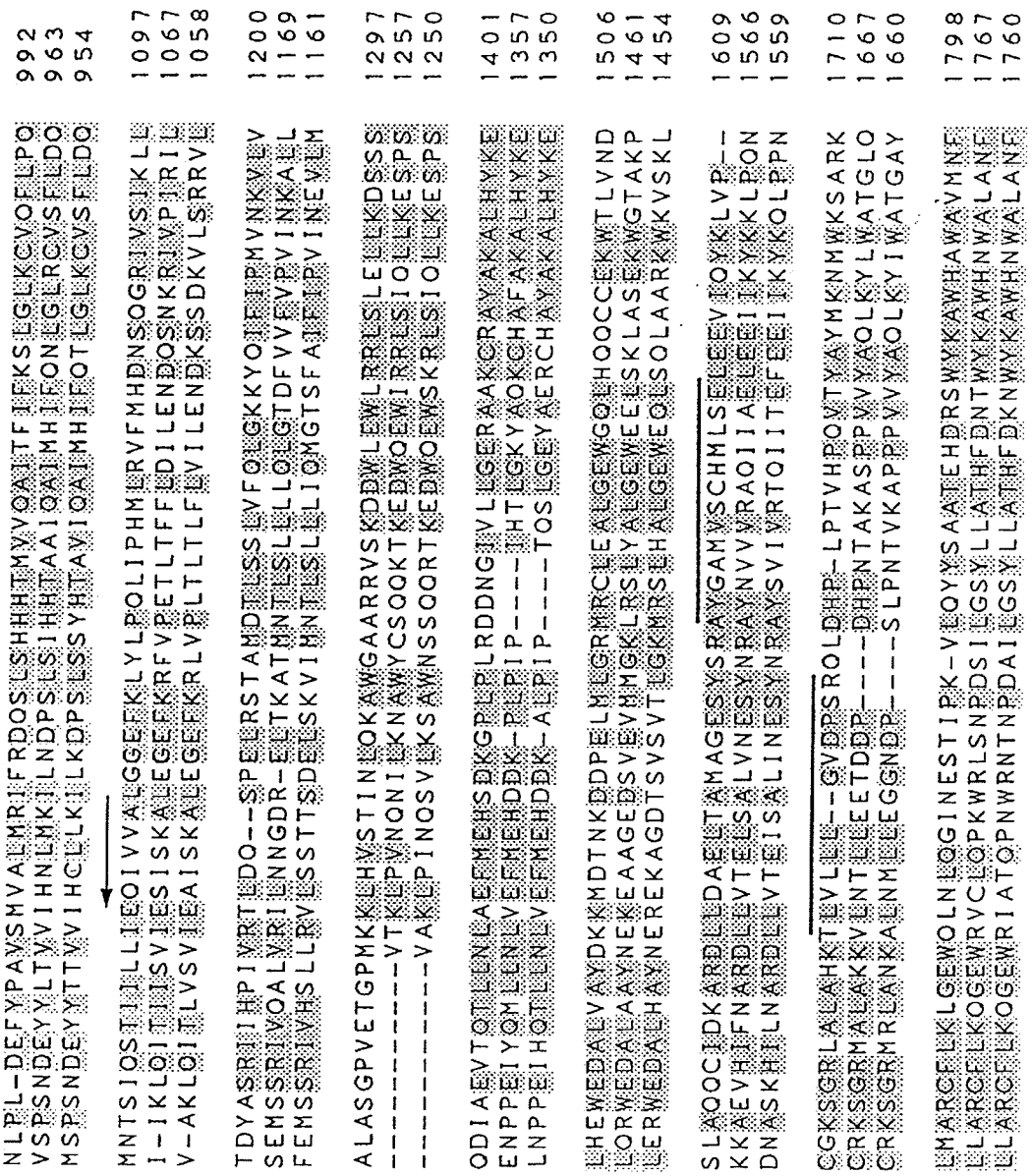


FIG. 4D

FIG. 4E

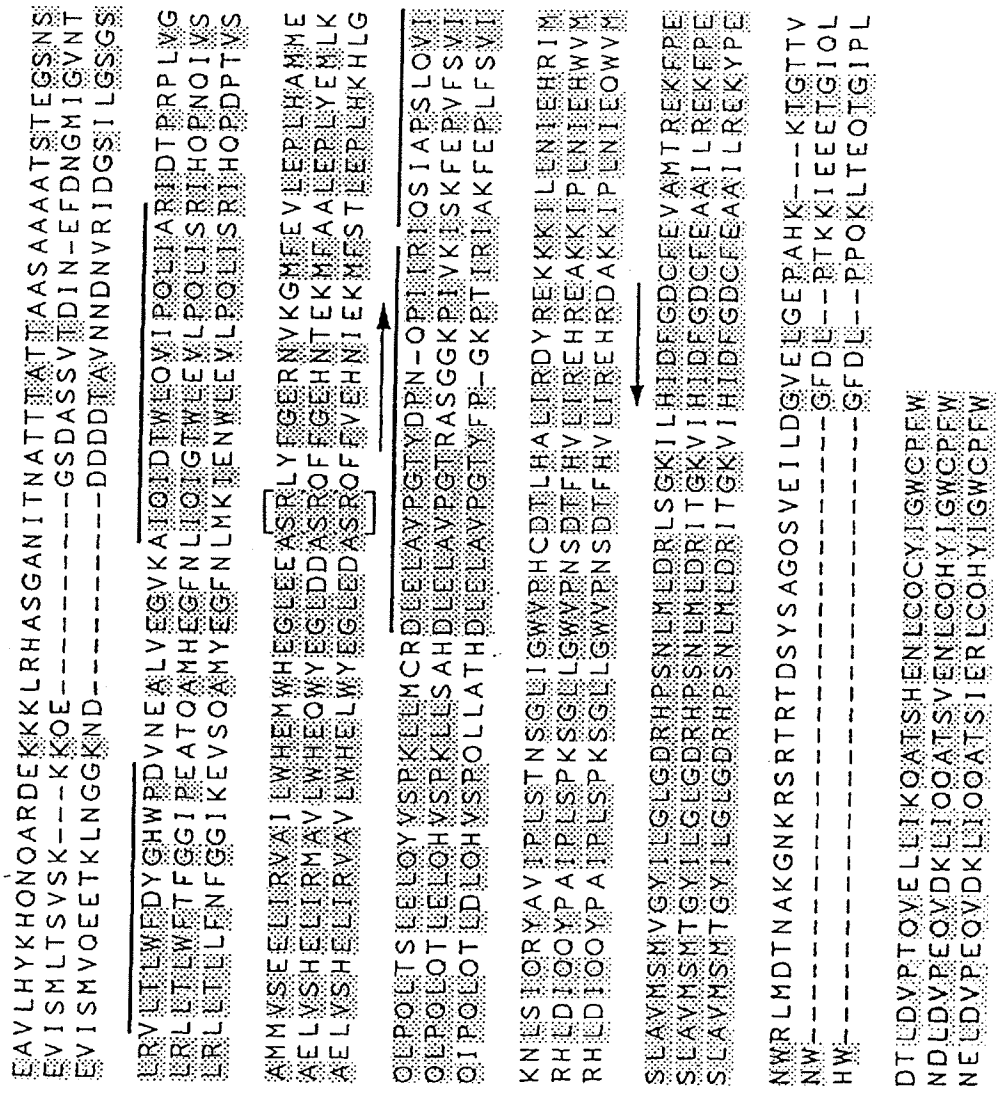


FIG. 4F

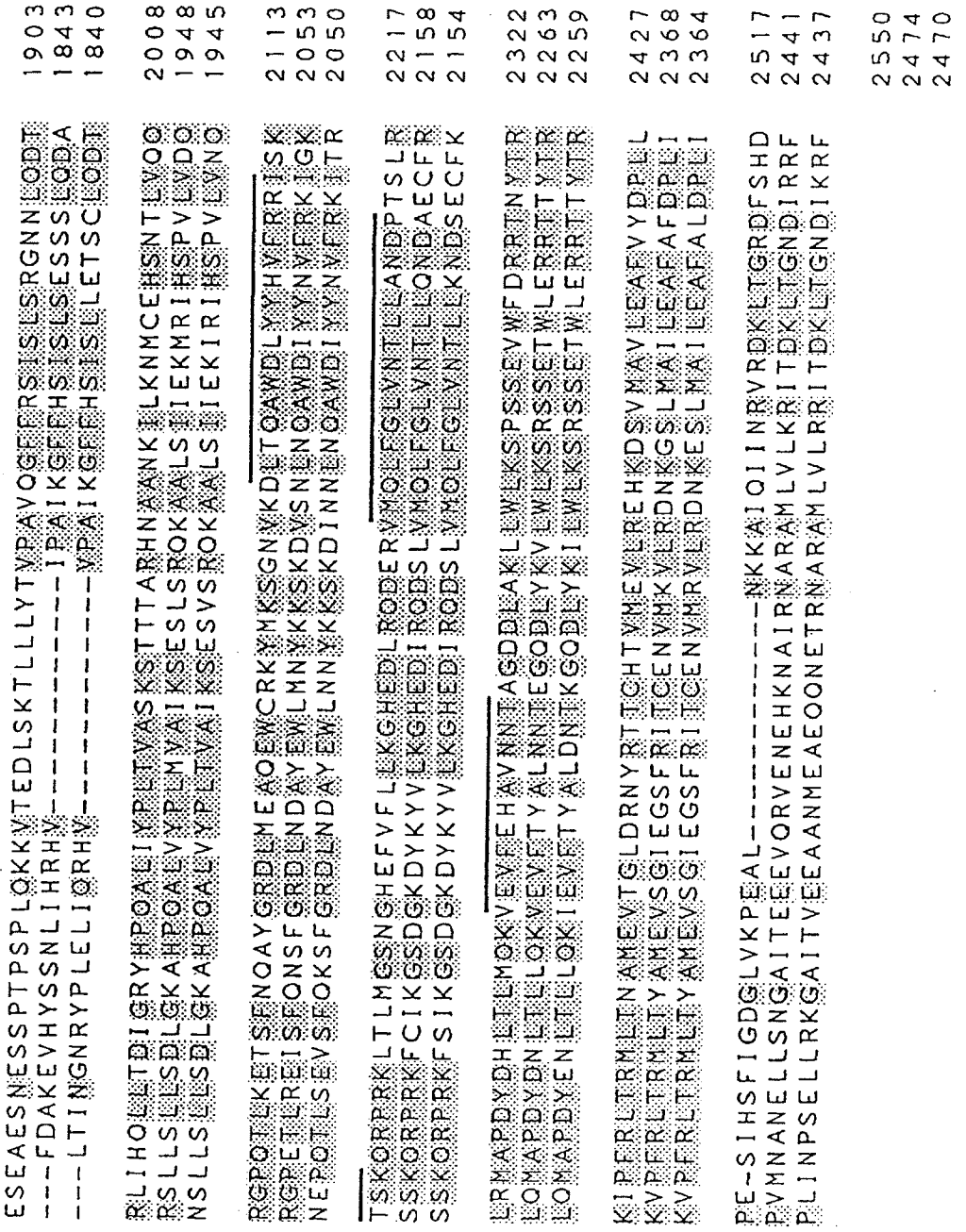
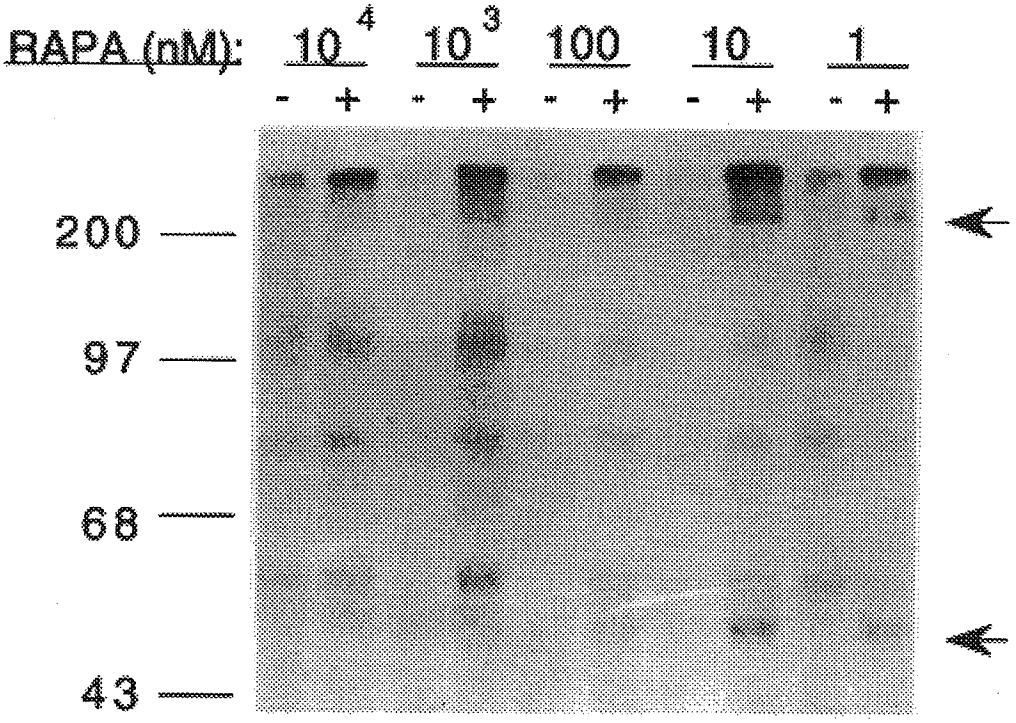


FIG. 5



MAMMALIAN PROTEINS THAT BIND TO FKBP12 IN A RAPAMYCIN-DEPENDENT FASHION

This application is a continuation-in-part of application Ser. No. 08/265,967, filed on Jun. 27, 1994.

This invention was made with government support under MH18501, DA00266, and DA00074, awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The natural products cyclosporin A, FK506, and rapamycin are potent immunosuppressants with realized or potential clinical applications in the prevention of graft rejection after organ transplantation and the treatment of autoimmune disorders (Borel, 1986; Kino et al., 1987; Martel et al., 1977). These drugs act by inhibiting intermediate steps in the signaling pathways that effect the T-cell response to antigen (for reviews see, Fruman et al., 1994; Kunz and Hall, 1993; Liu, 1993; Schreiber, 1991). This makes them useful probes for identifying the components of those pathways and determining their physiological roles.

The immunosuppressants interact with the immunophilins, which are small, soluble, receptor proteins that mediate their actions. Cyclosporin A (a cyclical undecapeptide) binds to cyclophilin A, whereas FK506 and rapamycin (two related macrolide antibiotics) bind to a distinct receptor protein, FKBP12 (Handschumacher et al., 1984; Harding et al., 1989; Siekierka et al., 1989). Though cyclophilin and FKBP12 differ markedly in amino acid sequence, both immunophilins have peptidyl-prolyl cis-trans isomerization (rotamase) activity, which is inhibited by their respective ligands (for review, see Heitman et al., 1992). However, this inhibition does not appear to explain the effects of the immunosuppressants (Bierer et al., 1990a, b; Tropschug et al., 1989). Instead, the action of cyclosporin A and FK506 derives from the binding of the drug-receptor complexes to the calcium-activated protein phosphatase, calcineurin (Liu et al., 1991). This association inhibits the catalytic activity of the phosphatase, which is required for the Ca^{++}-dependent initial step in the activation of the T-lymphocyte via the T-cell receptor (Flanagan et al., 1991; Kronke et al., 1984).

On the other hand, rapamycin appears to block a later, Ca^{++}-independent stage in the T-cell response. This drug selectively inhibits the IL-2 stimulated G1 to S cell-cycle transition that initiates T-cell proliferation (Dumont et al., 1990b). Although this inhibition has been correlated with the decreased activity of the 70 kDa S6 kinase ($pp70^{S6K}$), a known downstream effector of the IL-2 receptor, the FKBP12-rapamycin complex does not appear to interact directly with this kinase (Chung et al., 1992; Kuo et al., 1992). Similarly, in T-cells and other cell types, rapamycin blocks progression of the cell cycle by preventing the activation of the cyclin-dependent kinases $p33^{cdk2}$ and $p34^{cdc2}$, but an association of the drug-immunophilin complex with the kinases or their respective cyclins has not been demonstrated (Albers et al., 1993; Jayaraman and Marks, 1993; Morice et al., 1993).

In the budding yeast *S. cerevisiae,* rapamycin also causes an arrest in the G1 phase of the cell cycle through its binding to a highly conserved FKBP12 homologue (Heitman et al., 1991b). The toxicity of the drug for yeast cells has allowed, through genetic selection, the identification of two homologous genes, which, when mutated, render the cells

rapamycin-resistant (Heitman et al., 1991a). This led to the proposal that the products of these genes, which show some amino acid homology to the catalytic domain of the p110 subunit of PI-3 kinase, are the Targets Of Rapamycin and hence to the designation of the genes as TOR1 and TOR2 (Kunz et al., 1993). Direct support for this proposal, however, has not been presented and how the TOR gene products confer sensitivity to rapamycin remains to be elucidated. Alternatively, it has been suggested that in the signaling pathway blocked by rapamycin, the TOR proteins, like the S6 kinase and the cyclin-dependent kinases, lie downstream from the direct target of the FKBP12-rapamycin complex (Albers et al., 1993; Helliwell et al., 1994). This model assumes that the TOR mutations lead to the constitutive activation of the TOR1 and TOR2 proteins.

Besides binding to calcineurin in a FK506-dependent manner, FKBP12 can also interact with calcium-channel proteins, the ryanodine receptor, which mediates calcium induced calcium released (Jayaraman et al., 1992; Timerman et al., 1993) and the inositol 1,4,5,-triphosphate (IP_3) receptor (A. Cameron, A. Kaplin, D. Sabatini, J. Steiner, S. Snyder, unpublished). These associations do not require FK506 or rapamycin; indeed these drugs dissociate the FKBP12-channel complex.

There is a need in the art to identify, isolate, and purify the mammalian cellular proteins that interact with FKBP12 only in the presence of rapamycin. Such proteins play a role in immunological, neurological, and cell cycle functions.

SUMMARY OF THE INVENTION

It is an object of the invention to provide isolated, purified cDNA molecules encoding rapamycin and FKBP target molecules.

It is another object of the invention to provide fusion proteins comprising rapamycin and FKBP targets.

It is still another object of the invention to provide an isolated and purified rapamycin and FKBP target molecule.

It is still another object of the invention to provide an expression construct which directs synthesis in a cell of an RNA molecule which inibits expression of a rapamycin and FKBP target molecule.

It is yet another object of the invention to provide isolated, purified cDNA molecules which are complementary to genes encoding rapamcyin and FKBP target molecules.

It is an object of the invention to provide a method of screening for potential therapeutic agents.

It is another object of the invention to provide a method of purifying a rapamycin and FKBP target molecule.

It is still another object of the invention to provide a method of isolating DNA sequences which code for rapamycin and FKBP target molecules.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention an isolated, purified cDNA molecule is provided which encodes RAFT1, a protein having the amino acid sequence shown in SEQ ID NO:2.

In another embodiment of the invention a fusion protein comprising the amino acid sequence shown in SEQ ID NO:2 is provided.

In yet another embodiment of the invention an isolated and purified RAFT1 protein having the amino acid sequence shown in SEQ ID NO:2 is provided. Also provided is an isolated and purified RAFT2 protein, having an apparent molecular weight on SDS polyacrylamide gels of 35 kDa. Also provided is an isolated and purified mammalian RAFT

protein which is free of proteins which do not bind to rapamycin and FKBP12. Also provided is a mammalian RAFT protein prepared by the process of:

contacting a preparation of mammalian proteins with FKBP12 in the presence of rapamycin;

isolating mammalian proteins which bind to FKBP12 in the presence of rapamycin from those mammalian proteins which do not bind; and

dissociating bound mammalian proteins from FKBP12 to provide a mammalian RAFT protein.

In still another embodiment of the invention an expression construct is provided. The expression construct comprises a promoter operably linked to at least 20 nucleotides of the antisense strand of RAFT1 cDNA, said expression construct directing synthesis in a cell of an RNA molecule which is complementary to RAFT1 RNA.

In another embodiment of the invention an isolated, purified cDNA molecule comprising at least 20 nucleotides of the sequence as shown in SEQ ID NO:1 is provided.

In yet another embodiment of the invention a method of screening substances for potential as therapeutic agents is provided. The method comprises the steps of:

contacting a substance to be tested with three components: (a) FKBP12, (b) rapamycin, and (c) a protein selected from the group consisting of RAFT1 and RAFT2;

determining the amount of one of said components bound to the other components in the presence and absence of said substance; a substance which increases or decreases the amount of said component bound being a potential therapeutic agent.

In one embodiment of the invention a method of purifying a mammalian RAFT protein is provided. The method comprises the steps of:

contacting a preparation of mammalian proteins with FKBP12 in the presence of rapamycin;

isolating mammalian proteins which bind to FKBP12 in the presence of rapamycin from those mammalian proteins which do not bind;

dissociating bound mammalian proteins from FKBP12 to provide a mammalian RAFT protein.

In another embodiment of the invention methods of isolating mammalian RAFT DNA sequences are provided. One of the methods comprises:

probing a library of mammalian DNA sequences with a probe which comprises at least 15 contiguous nucleotides selected from the sequence shown in SEQ ID NO:1.

Another of the methods comprises:

amplifying a DNA sequence using at least one primer which comprises at least 10 contiguous nucleotides selected from the sequence shown in SEQ ID NO:1.

These and other embodiments of the invention provide the art with potent tools for identifying drugs useful in the treatment of immunological, neurological, and cell cycle-related diseases and defects.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows partial purification of the FKBP12-rapamycin target proteins from brain cytosol by heparin column chromatography. A cytosolic fraction prepared from a rat brain homogenate was applied to a heparin column. The material that remained bound to the column after washing with 5 column volumes of wash buffer containing 200 mM KCl, was eluted with a linear gradient from 200 mM to 600 mM KCl in homogenization buffer. Aliquots of the crude cytosol (S), the column flow through (FT), and the wash (W) were tested in the crosslinking assay with (+) or without (−) rapamycin (100 nM). Every other fraction eluted from the heparin column was tested in the crosslinking assay in the presence of 100 nM rapamycin. No rapamycin specific crosslinked products are visible in the crude cytosol because of the high concentrations of endogenous FKBP12 present in the initial sample.

FIG. **2** shows FK506 and unlabeled FKBP12 prevent the rapamycin-dependent association of ^{32}P-FKBP12 to the target proteins.

FIG. 2A) The heparin column eluate containing the RAFTs was tested in the crosslinking assay at the indicated concentrations of rapamycin with or without the addition of 1 μM FK506 or 1 μM FKBP12. FIG. 2B) Neither FK506 alone nor the ethanol vehicle induce crosslinking of FKBP12 to RAFT. The heparin eluate containing RAFT was tested in the crosslinking assay with the indicated concentrations of FK506 or ethanol. This experiment was repeated twice with identical results.

FIG. **3** shows purification of RAFT1 with a FKBP12-rapamycin affinity column. RAFT enriched fractions eluting from the heparin column between 300 and 450 mM KCl, were incubated in the presence (+) or absence (−) of 100 nM rapamycin with GST-(PKA)2-FKBP12 fusion protein (20 μg) immobilized on glutatlione agarose beads. The material that remained associated with the beads after extensive washes was analyzed by SDS-PAGE (8%) and silver staining. RAFT1 is present only in the sample treated with rapamycin. The large band at 36 kDa is the GST-FKBP12 fusion protein.

FIG. **4** shows the alignment of RAFT1 amino acid sequence with the predicted amino acid sequences of TOR2 (SEQ ID NO:4) and TOR1 (SEQ ID NO:3).

The alignment was maximized by introducing insertions marked by dashes. Sequences in RAFT1 identical to TOR2 and/or TOR1 are indicated with gray shading. The sequences of tryptic peptides obtained by microsequencing are indicated with a line above the RAFT1 sequence. Sequences used to design primers for PCR are indicated with an arrow above the residues (direction indicate sense or antisense). The PKC site conserved between RAFT1, TOR1 and TOR2 is boxed.

FIG. **5** shows rapamycin-dependent crosslinking of FKBP12 to two PC12 cell cytosolic proteins of approximate molecular weight 245 kDa and 35 kDa.

^{32}P-labeled FKBP12 (10^5 cpm) was incubated with cytosolic fractions from PC12 cells with or without the indicated concentration of rapamycin for 1 hr. at 4° C. The crosslinker DSS was then added and the incubation continued for 40 minutes before processing for SDS-PAGE (4%–12% gradient) and autoradiography. The arrows indicate the two Lands that appear only in the presence of rapamycin. This experiment was repeated three times with identical results.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have isolated and identified proteins, which we designate RAFT1 and RAFT2, that interact with the FKBP12-rapamycin complex. Rapamycin-induced binding of FKBP12 to RAFT1 occurs at drug concentrations as low as 1 and 10 nM, resembling pharmacological potency in vivo (Bierer et al., 1990a; Dumont et al., 1990a). FK506 and rapamycin bind with similar affinities to the same binding site on FKBP12 and antagonize each others' actions in vivo (Bierer et al., 1990a; Dumont et al., 1990b). Consistent with

these facts, FK506 does not induce interactions between FKBP12 and RAFT1 but, instead, prevents the rapamycin-mediated effect. Since rapamycin has pleiotropic effects on a wide variety of cell types, the target of its complex with FKBP12 is likely to be an early participant in several signal transduction pathways.

We have also isolated and purified a cDNA molecule which encodes RAFT1. The nucleotide sequence of RAFT1 is shown in SEQ ID NO:1. The predicted amino acid sequence of the protein, which exactly corresponds to the empirically determined amino acid sequences of tryptic peptides of RAFT1, is shown in SEQ ID NO:2. The cDNA sequence can be used to express in recombinant cells RAFT1 proteins or portions of the RAFT1 protein. Similarly, the cDNA sequence can be used to construct fused genes which will express fusion proteins comprising all or part of the RAFT1 sequence. Having provided the art with the amino acid sequence of the RAFT1 protein, other coding sequences can be devised which differ from that shown in SEQ ID NO:1 by virtue of the degeneracy of the genetic code. Such nucleotide sequences are within the scope of the present invention.

RAFT1 has an apparent molecular weight on SDS polyacrylamide gels of 245 kDa. RAFT2 has an apparent molecular weight on SDS polyacrylamide gels of 35 kDa. Isolated and purified RAFT1 protein can be obtained by means of recombinant DNA technology or by isolating and purifying the protein directly from natural sources. One means of purifying RAFTs involves contacting a preparation of mammalian proteins with FKBP12 in the presence of rapamycin. Those proteins which bind to FKBP12 in the presence of rapamycin can then be separated from those which do not bind. Bound proteins can then be dissociated to yield a preparation of a RAFT protein. It is convenient if the FKBP12 is immobilized, for example, on a solid support. One convenient means is to immobilize FKBP12 on a column-packing matrix. For example, an FKPB12-glutathione-S-transferase fusion protein can be readily bound to glutathione-agarose to provide immobilized FKBP12. Another means of purifying RAFT proteins is by use of a heparin chromatography column. The RAFT proteins bind to the heparin and can be eluted at 300 to 450 mM KCl.

Because of the role of rapamycin in immunological, cell cycle, and neurological functions, it may be desirable to inhibit the expression of RAFT1. One means to accomplish this is to use antisense polynucleotides. Antisense polynucleotides can be made synthetically, according to the sequence provided in SEQ ID NO:1. Alternatively, expression constructs may be used which comprise a promoter operably linked to at least 20 nucloetides of the antisense strand of RAFT1 cDNA. The expression construct directs the synthesis in a cell of an RNA molecule which is complementary to RAFT1 mRNA. Any suitable promoter can be used, depending on the cell system in which expression of the antisense molecule is desired.

The nucleotide sequence of SEQ ID NO:1 can be used to generate probes which comprise at least 15–20 nucleotides of the recited sequence. In some cases probes of 25, 30, 35, 40, 50, or 100 nucleotides may be desired. These probes can be used to screen a library of mammalian DNA molecules. Techniques for making nucleotide probes and screening genomic or cDNA libraries are well known in the art. Alternatively, other RAFT nucleotide sequences can be obtained by amplification of mammalian DNA using as primers one or two polynucleotides comprising at least 10 contiguous nucleotides selected from the sequence shown in SEQ ID NO:1. Techniques for amplification of DNA are also well known in the art.

RAFT1 and RAFT2 can be used to screen substances for potential as therapeutic agents for immunological, cell cycle, and neurological disease states. As described here, rapamycin, FKBP12, RAFT1, and RAFT2 bind to each other and form a complex. Test compounds can be screened for potential therapeutic utility by contacting a test compound with three components: (a) FKBP12; (b) rapamycin; and (c) a protein selected from the group consisting of RAFT1 and RAFT2. The amount of one of the components in the complex is determined, in the presence and in the absence of the substance to be tested. A substance which increases or decreasees the amount of the component in the comlex is a potential therapeutic agent. Means used for determining amounts of components can be any known in the art, including the use of radioactive components, antibodies specific for components, densitometry, etc.

EXAMPLES

The following materials were used in the examples described below. Frozen rat brains stripped of the meninges were obtained from Harlan Bioproducts (Indianapolis, Ind.). Other materials were purchased from the following sources: [γ-^{32}P]-ATP (NEG-02z) from New England Nuclear (Cambridge, Mass.), glutathione-agarose, heart muscle kinase (PKA, #P2645), and heparin-agarose from Sigma Chemical (St. Louis, Mo.), thrombin and antithrombin from Boehringer Mannheim (Indianapolis, Ind.), and disuccinimidyl suberate (DSS) from Pierce (Rockford, Ill.). Rapamycin was a gift of the Wyeth-Ayerst company (Philadelphia, Pa.) and FK506 a gift of the Fujisawa company (Tsukuba City, Japan).

Example 1

Rapamycin Promotes the Binding of FKBP12 to Two Cytosolic Proteins of Mr 245 and 35 kDa

A ^{32}P-radiolabeled FKBP12 probe was used to detect proteins that associate with the immunophilin in the presence of ligand, and are crosslinked to it by the bivalent reagent DSS. The probe was prepared by phosphorylating with [γ^{32}P]ATP a recombinant rat FKBP12 to which two consensus sites for cyclic AMP-dependent protein kinase (PKA) were added at the N-terminus (Blanar and Rutter, 1992; Li et al., 1992). Since this modification did not alter the capacity of the protein to associate with calcineurin in the presence of FK506, the probe can be used to identify a target of the FKBP12-rapamycin complex.

PC12 pheochromocytoma cell cytosolic extracts were incubated with ^{32}P-FKBP12 in the presence or absence of rapamycin and then treated with the crosslinker DSS before gel electrophoretic analysis followed by autoradiography. The drug caused the formation of two protein complexes with radioactive FKBP12, corresponding to bands of Mr 260 and 50 kDa (FIG. 5). Taking into account the 15 kDa Mr of the modified FKBP12 probe, the crosslinked proteins were estimated to be 245 kDa and 35 kDa, respectively. The crosslinked complexes were observed over a wide rapamycin concentration range, but were more prominent at the low concentrations of 1 and 10 nM, possibly because of an inhibitory effect on the association of the higher amounts of ethanol (the solvent of the drug) present at the higher drug concentrations (FIG. 5). Rapamycin also induced the formation of similar complexes when the probe was incubated with cytosolic extracts from several rat tissues, including

liver, kidney, heart, small intestine, thymus, testes, spleen and brain, but no significant differences in abundance of the crosslinked proteins between the tissues were observed. For convenience, further experiments were carried out with whole brain extracts.

The formation of the rapamycin-dependent complexes was specific for FKBP12, since in similar experiments with the related immunophilin ^{32}P-FKBP25, no ligand induced complexes were observed.

PC12 cells were maintained in culture as described (Altin et al., 1991). PC12 cells were lysed in homogenization buffer with 0.3% NP-40 instead of CHAPS. Lysis was accomplished in 2 ml buffer/T-150 flask by repeated vortexing at 4° C. Cell debris was sedimented by centrifugation for 10,000×g for 10 minutes at 4° C.

The labeled, cleaved FKBP12 was diluted to 10,000 cpm/ml in 50 mM Hepes pH 7.5, 1 mg/ml BSA. 10 μl of labeled protein (100,000 cpm total), 10 μl of tissue or PC12 cell extract, and 10 μl of drug dilutant buffer (20 mM Hepes 6.8, 100 mM KCl, 1 mM EGTA, 1 mM DTT) containing either 3-fold the desired final concentration of rapamycin, FK506, or equivalent amounts of ethanol, were mixed and incubated for 1 hour at 4° C. After this incubation, 1 ml of 5.5 mg/ml disuccinimidyl suberate (DSS) was added and the incubation continued for 40 minutes. The reaction was terminated by adding one column volume of 2× concentrated sample buffer (Laemmlli, 1970) containing 50 mM Tris pH 7.4 and processed by SDS-PAGE (10%, unless otherwise specified) and autoradiography.

Example 2

Specificity of the Rapamycin Induced Association: the Interaction of ^{32}P-FKBP12-Rapamycin with the 245 and 35 kDa Proteins is Competed by FK506 and by Unlabeled FKBP12

To investigate further the specificity of the interaction of ^{32}P-FKBP12-rapamycin with the cytosolic proteins, we performed a partial purification to remove endogenous FKBP12, which is present in brain at high concentrations (Steiner et al., 1992). This was accomplished by chromatography on a heparin column, to which the cytosolic proteins that interact with FKBP12-rapamycin bound and could be eluted at 300 to 450 mM KCl (FIG. 1). Free FKBP12, on the other hand, was recovered in the flow-through of this column, as demonstrated by binding to [^{3}H]FK506 (data not shown).

The rat brain extract was applied to a heparin column (2 ml of packed heparin-agarose per brain) at a flow rate of 1.5 ml/min. The column was washed with 10 column volumes of buffer (20 mM Hepes pH 6.8, 200 mM KCl, 1 mM EGTA, 50 mM NaF, 1.5 mM Na_3VO_4, 4 mM DTT, 1 mM PMSF) and the same protease inhibitors as in the homogenization buffer. The material bound to the column was eluted with a linear KCl gradient from 200 to 600 mM in homogenization buffer. Aliquots (10 μl) of the fractions collected were tested in the crosslinking assay and positive fractions were pooled and concentrated in a centriprep-100 (Amicon, Beverly, Mass.) to ⅓ starting volume. The flowthrough of the heparin column was assayed for the presence of FKBP with a ^{3}H-FK506 binding assay, as described (Steiner et al, 1992).

FK506 antagonizes actions of rapamycin, and both drugs compete for the same binding site on FKBP12 (Bierer et al., 1990a; Dumont et al., 1990a). Accordingly, we examined the influence of FK506 on the rapamycin-induced interaction of ^{32}P-FKBP12 with its putative cytosolic targets. At concentrations ranging from 1 nM to 1 μM rapamycin induced the appearance of intense bands representing crosslinked proteins and, at all rapamycin concentrations tested, this effect was antagonized by 1 μM FK506 (FIG. 2A). As expected for ligands of similar affinity for FKBP12, when equal concentrations (1 μM) of rapamycin and FK506 were present, the intensities of the crosslinked bands were reduced by approximately 50% and the reduction progressively increased with increasing ratios of FK506/rapamycin. The heparin column eluate apparently contains limiting amounts of the putative targets of the FKBP12-rapamycin complex, since excess unlabeled FKBP12 (1 μM) completely suppressed the appearance of the crosslinked bands containing labeled FKBP12 (FIG. 2A).

Control experiments (FIG. 2B) confirmed the specificity of the rapamycin effect since the formation of the complex was not induced by several concentrations of FK506 or by ethanol, the solvent of the drugs. These experiments demonstrate that the crosslinked proteins are specific targets of the FKBP12-rapamycin complex and not of the FKBP12-FK506 complex, nor of FKBP12 alone. Therefore, we designate the crosslinked proteins RAFT1 (245 kDa) and RAFT2 (35 kDa) for Rapamycin And FKBP12 Target.

We attempted to separate RAFT1 and RAFT2 under nondenaturing conditions by several chromatography and gel filtration procedures, including DEAE and CM cellulose, reactive dye green 5, and Superose 6 (data not shown). All of these efforts failed, suggesting that RAFT1 and RAFT2 are part of a complex, although it is possible that RAFT2 is a proteolytic fragment of RAFT1 that contains the FKBP12-rapamycin binding site and remains tightly bound to the rest of the polypeptide.

Example 3

Purification of RAFT1

We purified RAFT1 from the heparin column eluate based on its affinity for FKBP12-rapamycin. We constructed a glutathione-S-transferase-FKBP12 fusion protein by cloning, in frame downstream of GST, a cDNA encoding FKBP12 with two N-terminal PKA consensus sites (Smith and Johnson, 1988; Blanar and Rutter, 1992; Li et al., 1992). The encoded protein was expressed in bacteria, purified and immobilized on glutathione-agarose beads. SDS-PAGE analysis of the beads recovered after incubating them with the heparin eluate in the presence or absence of rapamycin shows that the drug induces the binding to the beads of a protein of 245 kDa (FIG. 3). With this simple purification scheme we were able to purify about 5 μg of RAFT1. A low transfer efficiency to nitrocellulose membrane resulted in only 2.5 μg being available for protein sequencing, which corresponds to 10 picomoles of a protein of this size.

Standard techniques of molecular biology cloning were used as described (Sambrook et al., 1989) for the preparation of GST-$(PKA)_2$-FKBP12 and GST-$(PKA)_2$-FKBP25 fusion proteins, unless otherwise specified. All cDNAs obtained with the polymerase chain reaction were sequenced using the Sequenase kit (Amersham, Arlington Heights, Ill.). cDNAs for the rat FKBP12 and FKBP25 were obtained with the PCR using 5' and 3' primers to the corresponding human FKBP12 (Standaert et al., 1990) or FKBP25 (Jin et al., 1992) sequences. The cDNAs were cloned into pBluescript (Stratagene, La Jolla, Calif.).

A 5' primer (PKA-12-1 or PKA-25-1) encoding a BamHI site, two consensus PKA phosphorylation sites (Blanar and Rutter, 1992; Li et al., 1992), and the first 6 amino acids of

FKBP12 or FKBP25 was used with a 3' primer (PKA-12-2 or PKA-25-2) encoding an EcoRI site and the last 6 codons of FKBP12 or FKBP25 in a PCR with Vent Polymerase (New England Biolabs, Beverly, Mass.) using the rat FKBP cDNAs cloned in pBluescript as templates. The amplified DNA fragments were gel purified, digested with BamH1 and EcoR1 and cloned into the pGEX-2T vector (Pharmacia, Upsala, Sweden) that had been linearized with the same restriction enzymes. The resulting construct was used to transform BL21 (DE3) *E. coli* (Novagen, Madison, Wis.) in which expression can be induced with IPTG.

The primer sequences were as follows:

PKA-12-1: 5' CCGGATCCCGTCGAGCTTCAGTTGAACTACGGCGTGC TTCTGTAGCCATGGGAGTGCAGGTGGA 3' (SEQ ID NO:5)

PKA-12-2: 5' GGCCGGAATTCTCATTCCAGTTTTAGAA 3' (SEQ ID NO:6)

PKA-25-1: 5' CCGGATCCCGTCGAGCTTCAGTTGAACTACGGCGTGC TTCTGTAGCCATGGCGGCGGCCGTTCC 3' (SEQ ID NO:11)

PKA-25-2: 5' GGCCGGAATTCTCAATCAATATCCACTA 3' (SEQ ID NO:12)

The fusion proteins were purified with glutathione-agarose as previously described (Smith and Johnson, 1988) from bacterial cultures induced with 1 mM IPTG.

The concentrated heparin column eluate was incubated for 2 hours at 4° C. with 1/50 volume of glutathione-agarose to remove endogenous glutathione binding proteins. The beads were removed by centrifugation at 1000×g for 3 minutes. Fresh glutathione-agarose (1/500 volume) and 20 μg of purified GST-PKA-FKBP12 fusion protein were then added to the cleared heparin column eluate with or without 100 nM rapamycin. After a 1 hour incubation at 4° C., the bead were washed 5× with 1.5 ml ice-cold PBS containing 1% Triton X-100 and 500 mM NaCl. The beads were transferred to 3× volume SDS-PAGE sample buffer, and the eluted proteins fractionated by SDS-PAGE and the gel silver stained.

Whether RAFT2 was also bound to the beads could not be determined in this experiment, because its presence would be masked by the large band of similar Mr corresponding to the GST-$(PKA)_2$-FKBP12 fusion protein. When smaller fusion proteins, such as an epitope-tagged FKBP12, were employed for the affinity matrix, the binding of the 35 kDa RAFT2 could also be observed.

The immunophilin fusion proteins containing N-terminal phosphorylation sites for PKA were labeled with a modification of published procedures (Blanar et. al., 1992, Li et. al., 1992). 10 ng of purified GST-PKA-FKBP12 or 25 was mixed with 40 units of PKA and 100 mCi of [γ-P^{32}]-ATP in a buffer containing 20 mM Hepes pH 7.7, 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT.

After a 1.5 hour at 37° C. the incubation mixture containing labeled fusion protein was dialyzed twice against 1L of thrombin cleavage buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$). The labeled fusion protein was cleaved by adding an equal volume of thrombin cleavage buffer containing 2 mg/ml thrombin and incubating at room temperature for 2 hours. The thrombin was inactivated by adding an equal volume of a stop solution consisting of 1 mM DTT, 1 mM PMSF, 100 units/ml antithrombin III. The specific activity of the probes was estimated at 1×10^5 cpm/pmol of the protein.

Example 4

Protein Sequencing of RAFT1: Homology to TOR1 and TOR2

Affinity purified RAFT1 was separated by SDS-polyacrylamide gel electrophoresis from other proteins that adsorbed to the glutathione-agarose beads, transferred to nitrocellulose membrane, and digested with trypsin. Fractionation of the tryptic digest by narrow-bore reverse phase chromatography yielded a complex pattern of over a hundred peaks whose purity was assessed by mass spectroscopy. In most cases, the peaks exhibited multiple mass to charge peak values and it was necessary to rechromatograph these peak fractions on a microbore columns of different selectivity.

For protein sequence analysis affinity purified material derived from 50 brains was fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The proteins transferred were visualized by Ponceau S staining, the 245 kDa RAFT1 band excised and processed for internal amino acid sequence analysis, essentially as described (Tempst et al., 1990).

Membrane-bound protein, about 2.5 μg, was subjected to in-situ proteolytic cleavage using 1 μg trypsin (Sequencing Grade; Boehringer-Mannheim) in 25 ml 100 mM NH_4HCO_3 (supplemented with 10% acetonitrile and 3% Tween-80) at 37° C. for 3 hours. The resulting peptide mixture was reduced and S-alkylated with, respectively, 0.1% β-mercapto ethanol and 0.3% 4-vinyl pyridine, and fractionated by two-dimensional reversed phase HPLC.

For the primary separations, a 2.1 mm Vydac C4 (214TP54) column was used with gradient elution at a flow rate of 100 μl/min. HPLC solvents and system configuration were as described (Tempst et al., 1990), with improved dead volume reduction through the use of glass capillary tubing (Elicone and Tempst, unpublished). Identification of Trp-containing peptides was done by manual ratio analysis of absorbances at 297 and 277 nm, monitored in real time using an Applied Biosystems model 1000S diode-array detector (Tempst et al., 1990). Fractions were collected by hand, kept on ice for the duration of the run and then stored at −70° C. before repurification and/or analysis. An enzyme blank was done on an equally sized strip of nitrocellulose cut from a blank area of the same blot. Repurifications (second dimension LC) were carried out on a 1.0 mm SGE ODS-2 C18 column using the same solvent system but at a flow rate of 30 μl/min. (C. Elicone, M. Lui, S. Geromanos, H. Erdjument-Bromage, P. Tempst, in press). Samples were always acidified (20% TFA final concentration) and then diluted twofold with 0.1% TFA before rechromatography.

Sequences of 23 peptides separated in this fashion were determined by a combination of automatic Edman degradation, matrix-assisted laser desorption mass-spectroscopy, and UV spectroscopy.

Peak fractions over background were analyzed by a combination of automated Edman degradation and matrix-assisted laser-desorption (MALDI-TOF) mass spectrometry (Geromanos et al., 1994; Elicone et al., 1994). After storage, column fractions were supplemented with neat TFA (to give a final concentration of 10%) before loading onto the sequencer disc and mass spectrometer probe tips. Peptide mass analysis (on 2% aliquots) was carried out using a model LaserTec ResearcH MALDI-TOF instrument (Vestec), with a 337 nm output nitrogen laser and 1.2 m flight tube. The matrix was a-cyano-4-hydroxy cinnamic acid, and a 28 kV ion acceleration and 4.3 kV multiplier voltage were used. Laser power and number of acquisitions were adjusted as judged from optimal deflections of specific maxima, using a Tektronix TDS 520 digitizing oscilloscope. M/z (mass to charge) spectra were generated from the time-of-flight files using GRAMS data analysis software. Every sample was analyzed twice, in the presence and

absence of a calibrant (25 femtomoles APID), as described (Geromanos et al., 1994). Chemical sequencing (on 95% of the sample) was done using a model 477A instrument from Applied Biosystems (AB). Stepwise liberated PTH-amino acids were identified using an "on-line" 120A HPLC system (AB) equipped with a PTH C18 (2.1×220 mm; 5 micron particle size) column (AB). Instruments and procedures were optimized for femtomole level phenylthiohydantoin amino acid analysis as described (Tempst and Riviere, 1990; Erdjument-Bromage et al., 1993).

Peptide average isotopic masses were summed from the identified residues (including the presumed ones) using ProComp version 1.2 software (obtained from Dr. P. C. Andrews, University of Michigan, Ann Arbor, Mich.). Peptide sequences were compared to entries in various sequence databases using the National Center for Biotechnology Information (NCBI) BLAST program (Altschul et al. 1990). Lower stringency alignments between all peptides and selected proteins were done using the Lipman-Pearson algorithm, available in the 'Lasergene' software package (DNASTAR).

Several protein sequence databases (PIR, SwissProt, translated Genbank) were searched for sequences that match any of the 23 peptide sequences obtained from microsequencing of RAFT1. While sequence similarities with hundreds of different proteins were obtained for many of the 23 peptides, none perfectly matched with any of the entries in the databases, nor did any protein match more than one or two peptides, other than the yeast proteins TOR1 and TOR2 (Kunz et al., 1993). Strikingly, sixteen out of the 23 peptides of RAFT1 could be aligned with the yeast TOR sequences, with varying degrees of similarity (FIG. 4).

Example 5

Molecular Cloning of RAFT1

To generate a probe for isolating a RAFT1 cDNA two degenerate oligonucleotides were used in a mixed oligonucleotide polymerase chain reaction (PCR) (Gould et al, 1989) with rat brain cDNA as template. The sense primer was made to a peptide sequence (TYDPNQP, SEQ ID NO:7) obtained from microsequencing of RAFT1, while the antisense primer corresponds to a sequence (HIDFGD, SEQ ID NO:8) conserved between TOR1, TOR2, and p110 PI-3 Kinase. From the alignments of the RAFT1 peptides to the TORs, this sequence was expected to be 220 amino acids downstream of that encoded by the sense primer. The predicted 660 bp PCR product was obtained, cloned, and its authenticity was verified by DNA sequencing, which showed that it encoded two other sequenced tryptic peptides. The PCR product was, therefore, used as a probe (3' probe) to screen a rat striatum cDNA library, which yielded a 5.5 kb partial cDNA clone. An antisense oligonucleotide to the extreme 5' end of this cDNA was then used in a PCR reaction with a degenerate sense oligonucleotide to another peptide sequence (NDQVFE, SEQ ID NO:9) obtained from microsequencing. The predicted 1.1 kb PCR product was obtained, cloned and used as probe (5' probe) to screen a rat brainstem cDNA library in parallel with the original 3' probe. Phage plaques that hybridized with both probes were isolated and one was found to carry a 8.6 kb insert. A degenerate sense oligonucleotide corresponding to the amino acid sequence TYDPNQP, which was obtained from microsequencing of RAFT1 and aligns to residues 2086 to 2093 of TOR2, and a degenerate antisense primer corresponding to amino acids 2296 to 2301 (HIDFGD, SEQ ID NO:8) of TOR2 were used in a PCR reaction with rat whole brain cDNA as template. The protocol for the PCR was: an initial 5 min at 94° C., followed by 35 cycles of 94° C. for 40s, 56° C. for 1 min, 72° C. for 1 min, and a final incubation at 72° C. for 5 min. The PCR products were fractionated on a 1.1% agarose gel, the expected 700 bp DNA fragment purified and subcloned into pBluescript.

The RAFT-1 cDNA fragment in pBluescript was amplified by PCR, the product gel purified and labeled by nick translation with a commercial kit (Boehringer Mannheim). This probe (designated 3' probe) was used to screen 1×10^6 phage plaques of a rat striatum λ ZAP library (Stratagene), as described (Sambrook et al.). Forty seven positive clones were identified and 10 of them were purified by an additional two rounds of screening. None of the inserts contained a complete open reading frame. The 5' end of the largest insert (5.5kb) was used to design a 18 bp antisense oligonucleotide (3.1 as) that was used in another PCR reaction with rat whole brain cDNA as template and a degenerate oligonucleotide corresponding to the amino acid sequence NDQVFE (SEQ ID NO:9 part of a peptide obtained from microsequencing) as the sense primer. The PCR products were fractionated on a 1% agarose gel and a DNA fragment of 1.1 kb isolated and cloned into the vector pCR-II using the TA cloning kit (Invitrogen, San Diego, Calif.). The cDNA fragment was amplified by PCR, the product gel purified and labeled by nick translation. This probe (designated 5' probe) was used to screen 1×10^6 phage plaques from a rat brainstem λ ZAP library. Duplicate filters were screened with the 3' probe. Eight clones hybridized with both the 5' and 3' probes, and five of these were purified through 2 additional rounds of screening. One clone contained a 8.6 kb insert that encodes all 23 peptide sequences obtained by microsequencing.

PCR primer sequences were as follows:

TYDPNQP (SEQ ID NO:7): 5'-GGGGGATCCACNTA (C/T) GA(C/T)CCNAA(C/T) CA(A/G)C-3' (SEQ ID NO:13)

HIDFGD (SEQ ID NO:8): 5'-GCGGAATTC(G/A) TCNCC(G/A)AA(G/A)TC(T/G/A) AT(G/A)TG-3' (SEQ ID NO:14)

NDQVFE (SEQ ID NO:9): 5'-GGGGGATCCAA(C/T) GA(C/T)CA(G/A)GTNTT (T/C)GA-3' (SEQ ID NO:15)

3.1as: 5'-GAGCCACCACGATTTGCT-3' (SEQ ID NO:10)

cDNA clones were sequenced using the flourescent terminator method of cycle sequencing on a Applied Biosystems 373a automated DNA sequencer at the DNA analysis Facility of the Johns Hopkins University (Smith et al., 1986; McCombie et al, 1992), or with the dideoxy chain termination method using the Sequenase kit (Amersham, Arlington Heights, Ill.). Oligonucleotides used for sequencing were synthesized on an ABI 394 synthesizer following ABI protocols. DNA sequence data was analyzed using Sequencher software from Gene Codes (Ann Arbor, Mich.). Protein alignments were done with help from the e-mail service of the Computational Biochemistry Research Group (CBRG) at the ETH.

This cDNA contains an open reading frame of 7.6 kb with an initiation methionine codon that conforms to the Kozak consensus sequence (Kozak, 1986) and is preceded by an in-frame termination codon. The protein encoded by this open reading frame contains all 23 peptide sequences obtained by microsequencing of RAFT1 (FIG. 4). Interestingly, none of the peptides sequenced correspond to the C-terminal 250 amino acids of RAFT1, which may indicate that this portion of the protein was proteolytically removed during the purification.

The RAFT1 cDNA predicts a protein of 2550 amino acids with a molecular mass of 289 kDa and a pI of 6.8. Over its entire sequence RAFT1 is 43% identical to TOR2 and 39% identical to TOR1 (FIG. 4). The C-terminal 600 amino acids of RAFT1, which, by analogy to the TORs (Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994), is predicted to contain lipid kinase activities, is 65% identical to the yeast proteins. The RAFT1 protein has over 20 consensus sites for phosphorylation by protein kinase C (PKC), including one at serine$_{2035}$, which is in the analogous position to the serine (S_{1972} in TOR1 and S_{1975} in TOR2) found mutated to arginine in rapamycin resistant yeast (boxed residues in FIG. 4).

The predicted RAFT1 protein is 80 amino acids longer than the TOR proteins, and contains several regions with no apparent homology to the yeast proteins, the largest being a 93 amino acid insertion corresponding to residues 270 to 363 of RAFT1. It is possible that these regions are generated by alternative splicing of exons that may be tissue specific to the brain. They are unlikely to be the translation product of unspliced introns because they were found in several cDNA clones isolated from different libraries and the DNA sequence does not reveal consensus splice junction sites.

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